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PROGRAM #PP1110

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SERDP ANNUAL TECHNICAL REPORT
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PROJECT TITLE:
Genetic Enhancement of an Anti-Freeze Protein for use as a Substitute for Ethylene Glycol for Aircraft Anti-icing

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PROJECT BACKGROUND:
Traditional anti-icing agents are either propylene or ethylene glycol. Glycols are effective in lowering the freezing point of water mixtures by the phenomenon of freezing point depression based solely on the molal concentration. The key environmental concerns with respect to use of ice control fluids are BOD loading and toxicity (human/mammalian and aquatic) resulting in extensive costs associated with the collection and cleanup associated with their use. For example, at Griffith AFB, NY, the use of glycols as a deicing fluid for aircraft has resulted in ground water cleanup programs costing over $8.2M. Additionally, an Air Force policy has been issued banning future purchase of ethylene glycol.

Traditional Colligative Freezing Point Depression.

Glycol based anti-icing agents are effective in lowering the freezing point of water mixtures by the phenomenon of freezing point depression. The magnitude of the freezing point depression of a given material is dependent on the amount of the material (solute), present in the solution (water) but not on its nature, and therefore freezing point depression is called a "colligative property", denoting "depending on the collection".

Under normal circumstances the ability of an antifreeze agent to depress the freezing point of a liquid is based solely on the molal concentration of the given material. That is one mole equivalent of any colligative deicing chemical will have approximately the same effect on the freezing point depression for a given quantity of water. In the case of an ionic salt such as sodium chloride the freezing point depression effect is two times the molal concentration of the NaCl. In the case of a salt such as calcium chloride ($CaCl_2$) the freezing point depression is three times the predicted value for the molal concentration of the salt since three moles of ions are generated for each mole of salt. The melting point and freezing point depression is directly related to the molal concentration of the solute. Although glycols lower the freezing point of water based on colligative effects water/glycol mixtures also exhibit a eutectic freezing point that is lower at a concentration of 75 percent than with pure glycol. Therefore aircraft hold-over times during icing conditions are longer with a 75/25 percent mixture of glycol:water than if the glycol were applied in a 100% concentration.

This colligative phenomenon is correlated to the effect of the solute molecule on the vapor pressure of the solution. Calculations are based on the assumptions that K_f for water is -1.86° C/mole over the entire range of concentrations, the salts are 100% ionic in solution, and the ions act independently of one another in their effect on the freezing point of the solution. This phenomenon is correlated to the effect of the solute molecule on the vapor pressure of the solution.

In theory, the depression of the freezing point in ideal solutions of non-electrolytes brought about by the addition of a solute, is proportional to the molal concentration of the solute and is independent of the nature of the solute according to the relationship shown below.

$$DT_f = K_f \times m$$

where DT_f is the freezing point depression, K_f is the molal freezing point lowering of the solvent (1.86°C for water), and m is the molality of the solution (moles of solute / 1000 grams of water).[1]

Non-Colligative Freezing Point Depression: Inhibition Of Ice Formation By AFPs.

In the case of naturally occurring antifreeze proteins, the observed freezing point depression is *several hundred times* the predicted effect based on the standard colligative relationship. This phenomenon has been described by Dr. Arthur DeVries, from the University of Illinois, Champagne-Urbana, in a species of Antarctic winter flounder. The antifreeze capabilities of naturally occurring antifreeze proteins were first observed when it was noted that the blood of the Antarctic Winter Flounder, *Pseudopleuronectes americanus* remained unfrozen at supercooled temperatures. It was also noted that the observed freezing point depression could not be attributed to the effect of the dissolved electrolytes, NaCl, potassium, calcium, urea, glucose and free amino acids. Subsequent research determined the origin of the antifreeze capabilities of the blood to be a specific glycoprotein, and associated low molecular weight polypeptides. The large freezing point depression was determined to be non-colligative, i.e., not proportional to the molal concentration of the protein in the blood.

Aspen System's Anti-Freeze Protein.

Aspen Systems Inc. has reverse translated the amino acid sequence of the *Dendriodes canadensis* antifreeze protein, determined by Dr. John Duman, into the DNA sequence for the gene. We have synthesized this oligonucleotide DNA sequence and cloned it into the protein expressing yeast, *Pichia pastoris*. Having successfully isolated several high yield producing clones, we have induced the expression of the antifreeze protein by the yeast on a 10 liter scale. Samples of the protein have been purified and sent to Dr. Andrea Wierzbicki for testing of their antifreeze properties.

Aspen Systems' antifreeze protein produced by the *Pichia pastoris* yeast inhibits the formation of ice crystals as well as the Winter Flounder Type I AFP. The experiments to determine the freezing point depression of Aspen Systems' antifreeze protein are currently under way, and are expected to be in the range of the *D. canadensis* hemolymph of ~9°C, (the Winter Flounder Type I AFP has a freeze point depression of only ~1.9°C

PROGRAM OBJECTIVE:

The need to develop environmentally benign anti-icers is particularly urgent because of recently passed EPA regulation which are making the continued use of current anti-icers, (e.g. ethylene glycol & propylene glycol), which have a high biological oxygen demand (BOD), prohibitively expensive. These regulations require the construction of on-site collection and treatment facilities for the spent deicing chemicals. The immediate ramification of these regulations is that waste deicing fluid run will beclassified as a non-storm water discharge which must have a low BOD, and hence this discharge requires an individual permit if it cannot be eliminated. In addition, it will be neccessary to sample the storm water for deicing chemical content and develop a storm water pollution prevention plan. The implementation of collection and treatment facilities translates to significant cost for the Air Force. Therefore the timely introduction of a nontoxic, low BOD anti-icer is particularly urgent. Aspen Systems anti-icing agent is based on a naturally occuring protein that will be nontoxic and have a low BOD. When the cost of production of these proteins is calculated in conjunction with the lower management and litigation costs of their use, they will be a very economically viable and environmentally beneficial alternative to the current anti-icing agents. The production of an environmentally benign anti-icing agent by this program will be essential to the deicing of both civilian and military aircraft, because it eliminates the high costs and associated danger of environmental pollution from this essential area of aircraft safety.

In order to meet this challenge, this project proposes to produce novel anti-icing agents using naturally occurring antifreeze proteins, which have a very low BoD compared to the current agents. Initial research has indicated that *Dendriodes canadensis* protein found in insects produces a freezing point depression that is 300 to 500 times the predicted value based on its molal concentration due to non-colligative properties. We have proposed to genetically alter the *Dendriodes canadensis* antifreeze protein gene in order to enhance the freezing point depression capabilities and therefore increase its usefulness and value as an aircraft anti-icing agent.

TECHNICAL APPROACH:

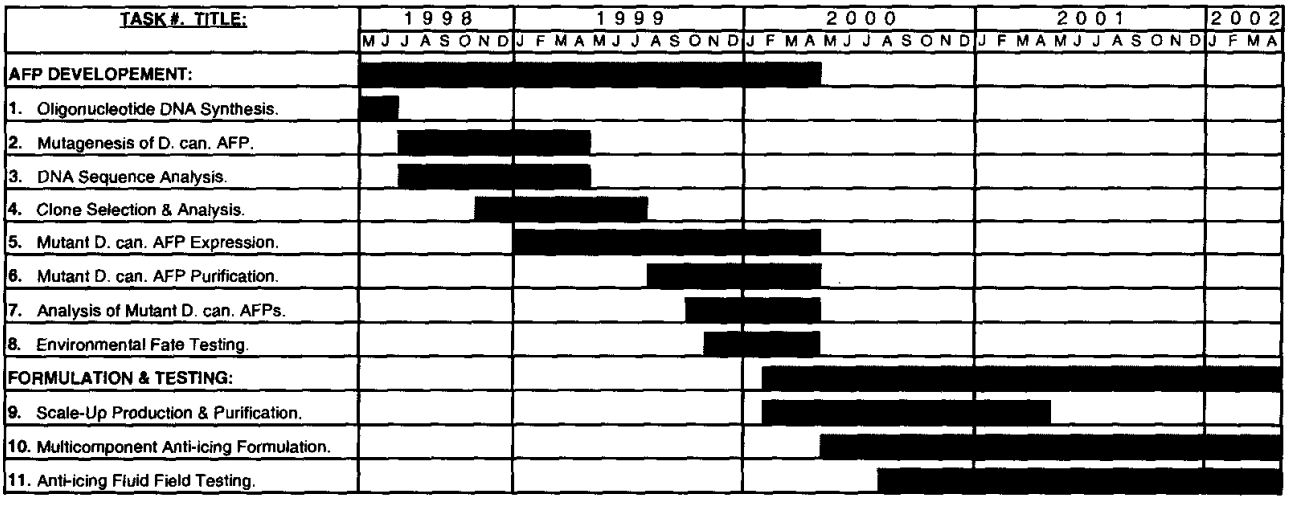
Aspen Systems proposed to genetically alter the gene of its proprietary antifreeze protein in order to enhance the freezing point depression capabilities of our Dendriodes canadensis Antifreeze Protein. This would increase its usefulness and value as a wing anti-icing agent. The first year of this program (FY99) was broken down into five tasks.

Task 1: DNA Oligonucleotide Synthesis
Task 2: Gene Mutagenesis & Cloning
Task 3: DNA Sequence Analysis
Task 4: Clone Selection & Analysis
Task 5: Mutant D. can. Protein Expression

Successful completion of the first year of this program was the cloning, selection and confirmation of the mutated antifreeze gene. We also completed the initial expression of several of these mutated Dendriodes canadensis antifreeze proteins. The purification as well as the continued enhancement of the expression conditions of the mutated proteins will occur within the second year of the program.

Successful completion of the second year of this program has been the expression and purification on a laboratory scale of the mutated antifreeze proteins. We will also send samples to the University of South Alabama for the determinations of their antifreeze protein properties. We will also send samples to EnviroSystems in order for environmental and toxicology properties to be determined These will be completed according to the task listed below.

Task 6: Mutant D. can. Protein Expression
Task 7: Analysis of Mutant D. can. AFPs.
Task 8: Environmental Fate Testing.
Task 9: Scale-up Production & Purification of the AFPs.



Task 1: *D. can.* AFP Amino Acid Sequence Analysis and Oligonucleotide DNA synthesis.

Upon selection of the amino acids to be altered by site-directed mutagenesis, oligonucleotides were designed, using the DNASIS program, based on criteria specified by the Altered Sites system, to introduce the new amino acid.

The DNA analysis program DNASIS Ver. 2.5 is a comprehensive nucleic acid and protein analysis software package. Including basic functions of restriction mapping, multiple alignment, and codon translations, plasmid map drawing, PCR primer design and RNA secondary structure with folding and energy predictions. It has the capability to do Internet BLAST Search for doing online sequence comparisons against standard sequence databases. It also has primer design tools including the analysis of hairpin turns, dimers, repeats, and false priming.

DNASIS includes the Higgins and Sharp algorithm for multiple sequence alignment; DNA and protein motif searching; proteolytic digest display; mutational site analysis; protein coding region prediction using Fickett's method; nearest-neighbor thermodynamic calculations for primer design; and a thorough manual with indexing and explanations of default parameters.

Sequence data can be input into DNASIS V2.S using Hitachi's Easy Reader backlit electromagnetic digitizer, our own data source or Hitachi's CD-DATA, (GenBank, EMBL, Swiss-Prot, NBRF-PIR). The DNASIS program includes the following capabilities;

Sequence Editing:

DNASIS also performs a wide range of input and editing functions on your DNA, RNA, or amino acid sequences, including; Primer Design, Translations, Subsequence Search, Voice Readback, Proofreading and Translation Editor.

Sequence Comparisons:

DNASIS contains a variety of alignment methods to compare and align multiple DNA and amino acid sequences and perform homology searches, including; Automatic Multiple Sequence Alignment, Manual Alignment, Higgins Algorithm, Dot Matrix Plots and Phylogenetic Trees.

Database Searches:

Using either customized CD versions or on-line access to sequence databases, DNASIS for Windows can rapidly search or compare sequences. Hitachi's CD-DATA includes presorted data for high speed searches. The BLAST software module enables the user to query the NCBI database as well as GenBank, Swiss-Prot, NBRF-PIR and EMBL on-line.

DNA Structure:

DNASIS offers a wide range of DNA Primary and Secondary structure functions. Restriction enzyme analysis is quick and easy. Searches can be customized by table cut type, and number of cuts. DNASIS also produces circular or linear maps and includes a Plasmid Map Drawing program. It also includes Customized Enzyme Tables, Enzyme Selection Criteria, Primer Design, RNA Folding and ORF Analysis

Protein Analysis:

DNASIS provides advanced protein analysis by using the latest protein structure prediction algorithms, including; Secondary Structure Prediction, Chou-Fasman, Robson, Helical Wheel

Graphics, Coiling Structure Graphics, Hydrophobicity, Amino Acid Content, Molecular Weight Determination as well as Reverse Translation to DNA.

Sequence Contingency:

The contingency manager manages DNA sequencing projects. DNASIS assembles an unlimited number of sequence fragments into a consensus sequence. It also allows for Automatic Fragment Assembly, Strategy View, Contingency Editing, Consensus Sequence and supports IUPAC Codes.

Primer Design:

DNASIS has advanced primer design functions including forward and reverse primers, and calculates Tm, AG%, and GC%. It also predicts Self Dimerization, Hairpin, Repeat Site and False Sites.

The oligonucleotide DNA synthesis of the primers for the site directed mutagenesis and the DNA sequencing will be done at Marshall University in Huntington, WV. Dr. Donald Primarano is the Director of the University's DNA Core Facility.

Task 2: Site-Directed Mutagenesis of the Dendroides canadensis AFP.

To ascertain the molecular prerequisites for AFP-ice binding, structural variants in which ice-binding residues are altered by recombinant DNA methods will be imployed. Techniques in computer - aided molecular design have given us insights for predicting the impact of such site-specific mutations on the protein's structure, and functionality. We will clone these site-specific mutations and determine their influence on the protein's antifreeze activity, including the ice-binding energy E_b, and the molecular dynamics of the antifreeze protein-ice interaction.

Site-directed mutagenesis is a valuable tool for the study of DNA function and protein structure and function. A number of different mutagenesis methods have been reported. Site-directed *in vitro* mutagenesis is accomplished by hybridizing a single stranded DNA (ssDNA) to a synthetic oligonucleotide which is complementary to the single-stranded template except for a region of mismatch near the center. It is this region that contains the desired mutation. Following hybridization, the oligonucleotide is extended with DNA polymerase to create a double-stranded structure. The nick is then sealed with DNA ligase and the duplex structure is transformed into an E. *coli* host.

The theoretical yield of mutants using this procedure is 50% (due to the semi-conservative mode of DNA replication). In practice, however, the mutant yield may be much lower, often only a few percent or less. This is assumed to be due to such factors as incomplete *in vitro* polymerization, primer displacement by the DNA polymerase used in the fill-in reaction, and *in vivo* host-directed mismatch repair mechanisms which favor repair of the unmethylated newly synthesized DNA strand. For this reason, the Altered Sites II Systems use antibiotic selection to increase the yield of mutants.

The Altered Sites II *in vitro* Mutagenesis System provides a high-efficiency procedure for generation and selection of oligonucleotide-directed mutants. This second generation system has several improvements over the original Altered Sites System, including the ability to mutagenize double-stranded template DNA, perform sequential rounds of mutagenesis without subcloning, and express the mutated gene products *in vivo* or *in vitro*.

This system uses antibiotic selection as a means to obtain a high frequency of mutants. The pALTER-1 Vector contains genes for ampicillin and tetracycline resistance, but the ampicillin resistance gene has been inactivated. The Ampicillin Repair Oligonucleotide provided restores ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide is annealed to the single- or double-stranded DNA template at the same time as the mutagenic oligonucleotide. Subsequent synthesis and ligation of the mutant strand links the two. The appropriate oligonucleotides can be used simultaneously in the mutagenesis reaction to inactivate one resistance gene while repairing the other. In this way, subsequent rounds of mutagenesis and selection can be performed on the same plasmid without subcloning.

Table 1. Properties of the pALTER-1 Vector.

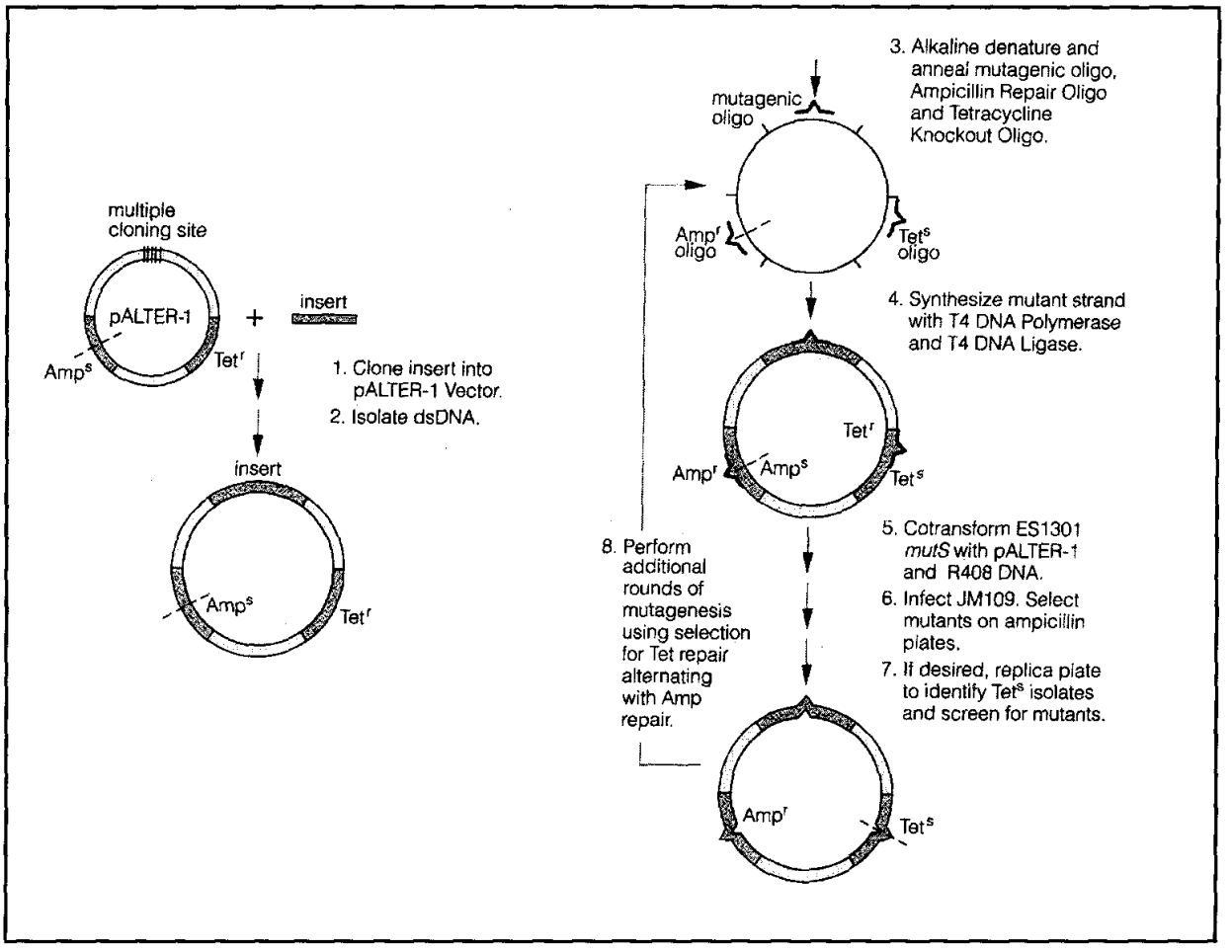
Selectable Markers [1]	*amp, tet*
Promoters Available	SP6, T7
Protein Expression *in vitro* and *in vivo*	No [2]
Compatible with ColE1 Plasmids	No
Blue/White Cloning	Yes

1.amp = ampicillin, let = tetracycline, cm = chloramphenicol

2. Expression is possible, but the cloned sequence must contain translation start sites.

Mutagenesis protocols are for both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). The mutagenesis reaction is initially transformed into a repair minus strain of E *coli* (ES1301 *mutS)* to avoid selection against the desired mutation. A subsequent strain transfer into JM109 ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants. The Altered Sites II Mutagenesis System allows consistently high mutagenesis frequencies (often >90%) using dsDNA or ssDNA.

Figure 1. Site-Directed Gene Mutagenesis of the AFP Gene.



The pALTER-1 Vector contains a multiple cloning site flanked by the opposing SP6 and T7 RNA polymerase promoters and inserted into the DNA encoding the lacZα-peptide. Cloning of a DNA insert into the multiple cloning site results in inactivation of the α-peptide. When plated on X-Gal indicator plates, colonies containing recombinant plasmids are white in a background of blue colonies. The SP6 and T7 promoters may be used to generate high specific activity RNA probes from either strand of the insert DNA. These sites also serve as convenient priming sites for sequencing of the insert. The pALTER-1 Vector carries gene sequences for both ampicillin and tetracycline resistance. However, the plasmid is ampicillin sensitive because a frame shift was introduced into this resistance gene by removing the *Pst I* site. Therefore, propagation of the plasmid and recombinants is performed under tetracycline selection. The pALTER-1 Vector also contains the f1 origin of replication, which allows for the production of ssDNA upon infection of the host cells with the helper phage R408 or M13K07. The pALTER-1 Vector has been used successfully with inserts up to 6kb in size.

Design of the Mutagenic Oligonucleotides.

The mutagenic oligonucleotide to be used in the site-directed mutagenesis must be complementary to the ssDNA strand produced by the mutagenesis vectors in the presence of helper phage. This is true for double-stranded DNA mutagenesis as well, since the mutagenic oligonucleotide

must hybridize to the same strand as the antibiotic repair oligonucleotide for the selection to be effective.

The stability of the complex between the oligonucleotide and the template is determined by the base composition of the oligonucleotide and the conditions under which it is annealed. In general, a 17-20 base oligonucleotide with the mismatch located in the center will be sufficient for single base mutations. This gives 8-10 perfectly matched nucleotides on either side of the mismatch. For mutations involving two or more mismatches, oligonucleotides 25 bases or longer are needed to allow for 12-15 perfectly matched nucleotides on either side of the mismatch. Oligonucleotides 26 and 27 bases long have been used successfully to perform four base insertions and deletions. Larger deletions require an oligonucleotide having 20-30 matched bases on either side of the mismatched region.

The annealing conditions required may vary with the base composition of the oligonucleotide. AT-rich complexes tend to be less stable than GC-rich complexes and may require a lower annealing temperature to be stabilized. Routinely, oligonucleotides can be annealed by heating to 75°C for 5 minutes followed by slow cooling to room temperature.

Select amino acids within the ice crystal binding domain will be changed to enhance the antifreeze activity of the *D. canadensis* protein. These substitutions will be accomplished by cloning the *D. can.* antifreeze protein gene into the pALTER-Exl vector from Promega. The *D. can.* antifreeze protein gene will be band isolated following restriction digestion, and a Nsi I site added to the 5' end by the polymerase chain reaction (PCR). The *D. can.* antifreeze protein DNA can then be ligated into the pALTER-Exl vector which contains a tetracycline (tet) resistance gene and amp sensitivity due to the point mutation in the amp resistance gene at Not I and Nsi I sites. This vector allows the simultaneous annealing of a mutagenic *D. can.* antifreeze protein o go, a repair amp gene oligo and a tet gene knockout oligo which are polymerized together into a new three point mutated DNA vector and insert. The mutated pALTER-Exl vector and RS08 DNA are used to cotransform E. coli JM107 cells. Switching from tet. resistance to amp. resistance allows for selection of the new mutated *D. can.* antifreeze proteins by replica plating on both amp. and tet. plates. After selection and confirmation by sequencing, the mutated *D. can.* antifreeze protein inserts will then be isolated and cloned into the *Pichia pastoris* expression vector.

Table 2. Amino Acid Selection for Point Mutating.

Sample	Parent D. can. A.A.	Mutated D. can. A.A.	Predicted Affect
28	Glycine	Cysteine	Conformational Change
37	Arginine	Threonine	Increase Ice Binding Affinity
42/45	Aspartic acid/Asparigine	Threonine/ Threonine	Increase Ice Binding Affinity
43	Cysteine	Glycine	Conformational Change
47	Proline	Threonine	Increase Ice Binding Affinity
52	Alanine	Deletion	Conformational Change
84	Tyrosine	Phenylalanine	Conformational Change
100	Alanine	Threonine	Increase Ice Binding Affinity
Mlu	No Restriction Site	Mlu I Restriction Site	Add Ice Binding Domain

Task 3: DNA Sequencing of the Mutated D. can. AFP Genes.

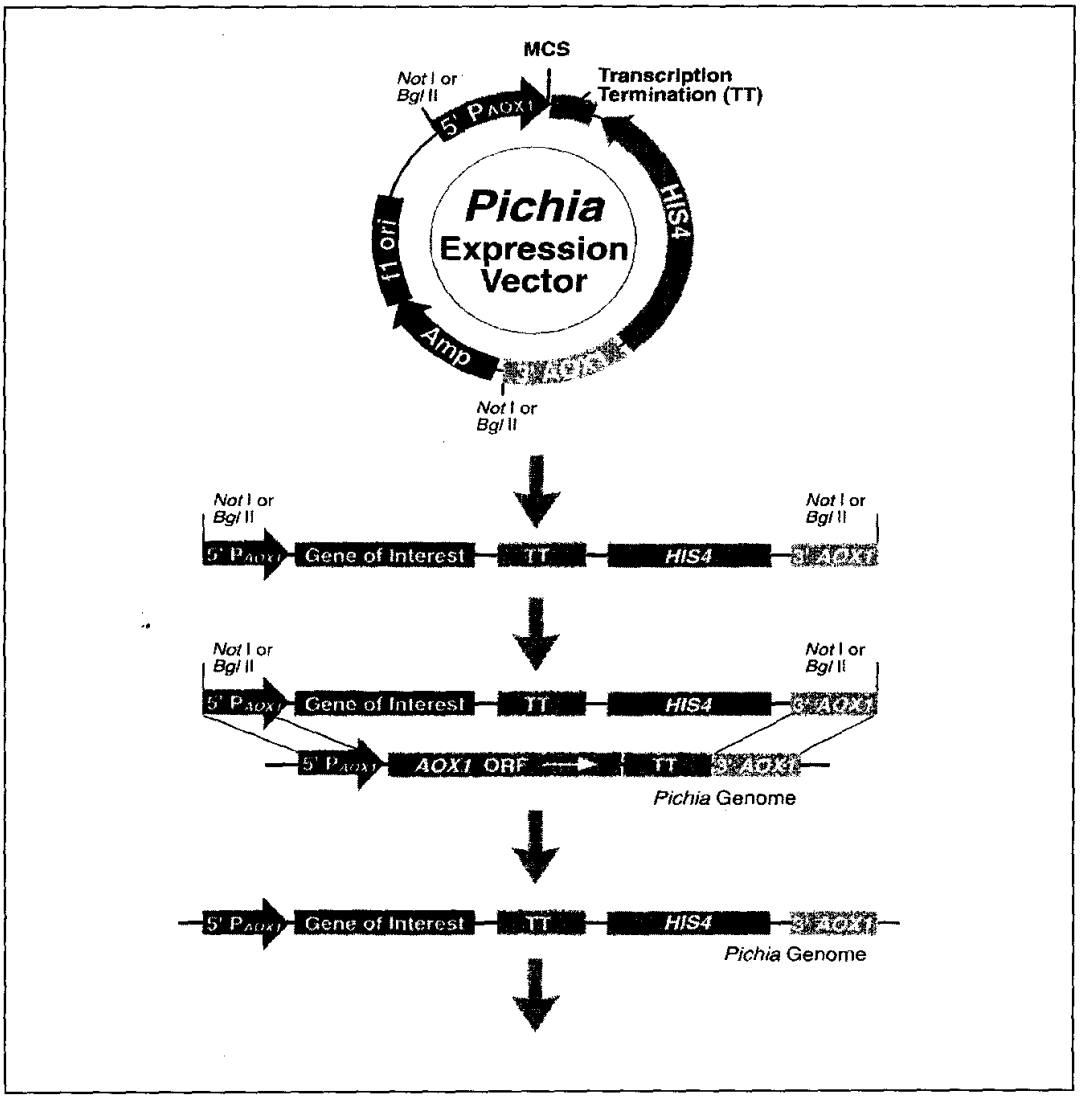
After each step in the specific site-directed mutagenesis of the *D. can.* antifreeze protein gene is completed, the DNAs will be sequenced. This sequencing will be completed after the mutagenesis reactions and cloning are completed as well as after the cloning of the mutated genes into the pHIL-S1 vector. DNA samples from the selected clones (with corresponding primers) will supplied to Mr. Edwin Rich, Director, at Boston University's Protein/DNA Research Center for sequencing by the ABI automated sequencing system.

The sequencing reactions are accomplished by polymerase chain reaction in the presence of flouresencently labeled reaction termination dideoxynucleotides. The protocol used follows the ABI, (Applied Biosystems Inc.) approved protocol for use with their automated DNA sequencing system which includes polyacrylamide gel electrophoresis and band flouresence detection system. The results are then automatically tabulated and stored for analysis.

Task 4: Yeast Cloning, Selection and Analysis.

The constructed genes for the mutated *D. can.* antifreeze protein will be ligated into the pHIL-S1 yeast expression vector containing the Pichia signal sequence. This ligated vector-gene DNA is used to transform DH5α E. coli cells. The transformants are screened, then clones selected for further analysis. Purified DNA samples from these clones are then isolated for sequencing in order to confirm the presence and orientation of the *D. can.* gene within the vector. DNA is also isolated and purified for use in the transformation of the Pichia pastoris yeast.

Figure 2. Yeast Protein Expression System.



Preparation Of Spheroplasts And Their Transformation.

In general, spheroplasting provides the highest efficiency of transformation for most researchers (103 to 104 transformants per ug DNA). *Pichia* can also be transformed using PEG 1000 or lithium chloride. These two protocols, particularly lithium chloride, perform less well than spheroplasting. Transformation in *Pichia* is less efficient than for Saccharomyces but still provides us with ample transformants.

The cell wall of yeast prevents the uptake of DNA. To enable yeast to take up DNA, it is necessary to partially remove the cell wall. Zymolyase is a 5-glucanase that hydrolyzes the glucose polymers with, B-1,3 linkages in the cell wall. Addition of Zymolyase partially digests the cell wall. It is critical not to overdigest the cell wall as doing so will cause the cells to lyse. Zymolyase digestion is monitored by the sensitivity of the cells to SDS. Aliquots of cells are added to SDS, lysing the spheroplasts. This causes a clearing of the solution which is monitored by the absorbance (light-scattering) at 800 nm. It has been empirically determined that when 30% of the cells have lysed (70% spheroplasting) that digestion is optimal.

Cells are then washed with an isotonic solution to remove the enzyme and incubated with DNA. The cells are resuspended in sorbitol to facilitate cell wall regeneration and plated. At the end of 4-6 days, His+ transformants will be seen on sample plates. Transformation efficiency was generally ~100 His+ transformants/ug of DNA using the spheroplast method. There were no colonies on the "No DNA" or pBR322 plate or the plasmid only (no cells) plate.

At this point, plates of His+ GS115 transformants are scored for Mut+ and Mut^S phenotype. Included in the kit are two strains which provide examples of Mut+ and Mut^S phenotypes. The GS115 Albumin is Mut^S and the GS115 Gal is Mut+.

The two control strains for background protein expression in Pichia are also isolated. One control is the parent plasmid linearized in such a way to generate His+ Mut^S transformants. The other control is the parent plasmid linearized to generate His+ Mut+ transformants .

Transformation of GS115 with Bgl II linearized pHIL-S1 constructs favor recombination at the AOXI locus. Displacement of the alcohol oxidase (AOXI) structural gene occurs at a frequency of 5-35% of the His+ transformants. By patching or replica-plating on Minimal Dextrose (MD) versus Minimal Methanol (MM) plates, Mut+ and Mut^S transformants were readily distinguished.

Because Mut^S transformants are not producing alcohol oxidase (the product of the AOXI gene), they cannot efficiently metabolize methanol as a carbon source and therefore grow poorly on minimal methanol (MM) medium. This slow growth on methanol can be used to distinguish His+ transformants in which the AOX1 gene has been disrupted (His+ Mut^S) from His+ transformnants with an intact AOXI gene (His+ Mut+).

Transformation of GS115 with Sal I linearized constructs favor recombination at the HIS4 locus. Most of the transformants should be Mut+; however, with the presence of the AOXI sequences in the plasmid, there is a chance that recombination will occur at the AOXI locus, disrupting the wild-type AOXI gene and creating His+ Mut^S transformants. Again, testing on MD and MM plates will allow you to isolate His+ Mut+ transformants.

In contrast to His+ Mut^S transformants generated by linearizing with Not I or Bgl II, most of the His+ transformants generated by digestion with Sal I, should be Mut+. These will be gene insertion events at either the His4 or AOXI loci, leaving an intact AOXI, locus.

Clones are then selected for each mutated D. can. gene of the Sal I and Bgl II digested DNA transformants for further phenotypic analysis. The phenotype analysis of these transformants based on their ability (or lack of), to grow on methanol containing plates demostrated the presence of many clones derived from pHIL-S1 that was digested with Sal I to give His^+ Mut^+ transformants, and several clones derived from Bgl II digested pHIL-S1 to give His^+ Mut^S transformants

PCR Analysis Of The Transformed Clones.

To analyze *Pichia* integrants to determine if the genes of interest have been integrated into the *Pichia* genome polymerase chain reaction was performed on isolated genomic DNA from Mut^S and Mut+ *Pichia* clones using the same protocol as well as isolated DNA from the strains transformed with the parent plasmid. After isolating the DNA, amplification of the gene of interest was carried out with the 5' AOXI primer paired with the 3' AOXI primer. This protocol is useful for confirming integration of the gene of interest but does not provide information on the site of integration.

Task 5: Expression of the Mutated *D. can.* Genes into the *Pichia pastoris* system:

Once we have isolated several Mut^S and Mut+ recombinant clones using the GS115 yeast strain which have been confirmed by PCR which contain our DNA inserts, the purpose of the next experiments is to determine the optimal method and conditions for expression of our gene. Below are some factors and guidelines which need to be considered before starting expression in *Pichia pastoris*. As with any expression system, optimal expression conditions are dependent on the characteristics of the protein being expressed.

Media.

We will be using BMGY/BMMY (buffered complex glycerol or methanol medium) for expression media. BMGY, and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of our protein. Because these media are buffered with phosphate buffer, a wide range of pH values may be used to optimize production of your protein. BMGY/BMMY contain yeast extract and peptone which may help stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone act as a "mixed feed" allowing better growth and biomass accumulation.

Proteases.

There are some proteins specifically susceptible to proteases that have optimal activity at neutral pH. If this is the case, expression using MGY and MM media may be indicated. As Pichia expression progresses in an unbuffered medium such as MM, the pH drops to 3 or below, inactivating many neutral pH proteases Pichia is resistant to low pH, so the low pH will not affect growth. In contrast, it has been reported that by including 1% Casamino acids and buffering the medium at pH 6.0, extracellular proteases were inhibited, increasing the yield of mouse epidermal growth factor.

If our proteins are especially susceptible to neutral pH proteases we may have to do our expressions in an unbuffered medium (MM). If there is no evidence that our secreted protein of interest is susceptible to proteases at neutral pH, we will do our initial expressions in BMMY. If the expressed protein is degraded, expression in an unbuffered medium will then be tried.

Aeration.

The most important parameter for efficient expression in Pichia is adequate aeration during methanol induction. As a general rule when inducing expression, cultures should not be allowed to be more than 10-30% of your total flask volume. Aeration is not as critical when generating biomass before induction.

Kinetics of Growth.

Note that while Mut+ and Mut^S strains will grow at essentially the same rate in YPD or glycerol media, Mut+ will grow faster than Mut^S when both are grown on methanol because of the presence of the AOXI gene product.

Temperature and Shaking.

All expressions are done at 30°C, in a shaking incubator. It is critical that the temperature does not exceed 30°C. We will be using a floor shaking incubator, shaking at 225-250 rpm.

Guidelines for Expression.

After we have verified our recombinants in GS115 as well as a control recombinant of GS115/Vector (no insert). When performing our expression, it is important to run the proper controls so that we will be able to interpret our expression results. The expression controls used are:

GS 115 / His+ Mut^S albumin - secretion control
GS 115 / Vector (no insert) - background control

Due to the fact that recombination can occur in many different ways which can effect expression (clonal variation), it is recommended that we test 6-10 verified recombinant clones and screen their expression levels. Starting with colonies from the freshest plates available is best because colony viability drops over time, or we may start the cultures from a frozen glycerol stock that was generated from a single colony.

The following steps are a guideline for expression but we may have to change the conditions to optimize expression for our protein. We will use 50 ml conical tubes to analyze a number of recombinants, then selected clones will be grown at larger volumes in side baffled flasks.

Protocol:

1. Using a single colony, inoculate 25 ml of BMGY in a 250 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches an OD600 = 2-6 (approximately 16-18 hours). The cells will be in log-phase growth.
2. Harvest the cells by centrifuging at 1500-3000 x g for 5 min at room temperature. Decant supernatant and resuspend cell pellet to an OD600 of 1.0 in BMMY, to induce expression (approximately 100-200 ml).
3. Place culture in a 1 liter baffled flask and return to incubator to continue growth.
4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.
5. At each of the times indicated below, transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2-3 minutes at room temperature. Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60,72 (3 days), 84, and 96 (4 days).
6. For secreted expression, transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at -80°C until ready to assay. Freeze quickly in a dry ice/alcohol bath.
7. Analyze the supernatants and cell pellets for protein expression by Dot Blot, SDS-PAGE and Western Blot.

Antibody Detection and Conformation of the Mutated AFPs.

In preparation for the analysis of the expression products of the cloned *Dendroides canadensis* AFP gene, Aspen Systems has previously contracted Bio-Synthesis Inc. located in Lewisville, TX to synthesize and purify two polypeptides for use as antigens for the production of polyclonal antisera. These peptides correspond to two of the beta turns on the exposed surface of the *D. canadensis* AFP protein. These peptides were used by Bio-Synthesis, as immunogens to illicit an immune response in rabbits.

	44	53
Peptide 1:	NH2-Gly-Asn-Cys-Pro-Asn-Ala-Arg-Thr-Ala-Cys-COOH	

98 107

Peptide 2: NH2-Ala-Thr-Ala-Cys-Thr-Asp-Ser-Thr-Gly-Cys-COOH

The polypeptides will be conjugated to Keyhole Limpet Hemocyanin (KLH) protein and then injected into two rabbits each. The isolated serum was tested for its ability to recognize the immunogen in an ELISA test. Positive antisera have been used in Dot blot and Western blot analysis of the induced *Pichia pastoris* media to test for the presence and size characterization of the *D. canadensis* AHP.

Antibody Capture on Nitrocellulose - Dot Blots.

In order to determine the presence of the mutated *D. can.* AFPs within the expression media, Dot blots are the assays of choice for semi-quantitative analysis. The antigen is bound directly to a nitrocellulose sheet, and then detected by AFP specific antibodies, discussed previously.

1. A expression media solution of at least 1 ug/ml is added to a nitrocellulose sheet at 0.1 ml/cm^2. The protein is allowed to bind to the paper for 1 hr. Higher concentrations of proteins will increase the signal and make screening faster and easier.
2. The nitrocellulose sheet is then washed three times in PBS.
3. The sheet is then placed in a solution of 3% BSA in PBS with 0.02% sodium azide for 2 hr to overnight. To store the sheet, wash twice in PBS and place at 4°C with 0.02% sodium azide, or shake off excessive moisture from the sheet, cover in plastic wrap, and store at -70°C.
4. Apply 1 ul of the anti-AFP hybridoma tissue culture supernatant to each square. Incubate the nitrocellulose sheet on the parafilm at room temperature in a humid atmosphere for 30 min.
5. Quickly wash the sheet three times with PBS, then wash two times for 5 min each with PBS.
6. Add rabbit anti-mouse immunoglobulin-HRP per 3-mm square in 3% BSA/PBS with 0.02% sodium azide (about 2.0 ml/cm^2).
7. After 30-60 min of incubation with shaking at room temperature, wash extensively with PBS.
8. In order to visualize the results colormetrically, cover the nitrocellulose with IBI's Enzygraphic Web, and record the results after 2 min.

Task 6: Mutant D. can. Protein Purification.

In order to purify the *D. can.* antifreeze protein from the crude fermentation media and cells in the most efficient and economical manner possible, Aspen Systems has determined that the expanded bed absorption chromatography technology (STREAMLINE) developed by Pharmacia Inc. has the greatest potential for accomplishing this goal.

The principle of expanded bed adsorption is that the bed is expanded by the upward liquid flow. Controlled, even flow is assured by the design of the liquid distribution system in STREAMLINE columns. Adsorbent particles are suspended in equilibrium due to the balance between particle sedimentation velocity and upward flow. The defined particle distribution and high density of STREAMLINE adsorbents yield a stable, uniformly expanded bed; smaller, lighter particles in equilibrium in the upper part of the column and larger, heavier particles in the lower part. There is no back-mixing and the expanded bed is ready for efficient adsorption.

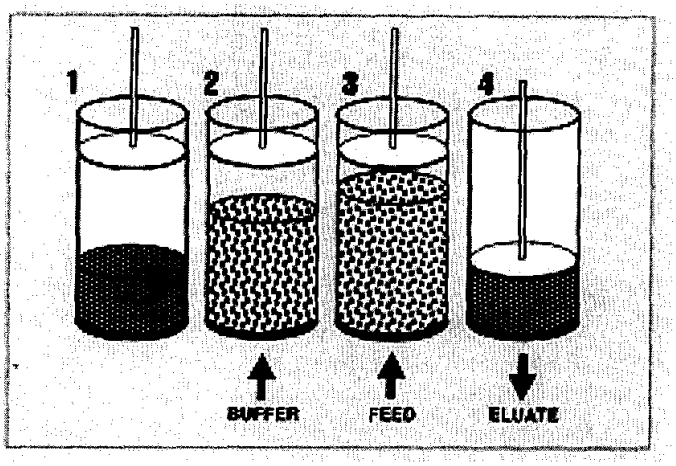


Figure 3. Principles of Expanded Bed Operation

Crude, unclarified feed is applied to the expanded bed with an upward flow. Target proteins are captured on the adsorbent while cell debris, cells, particulates and contaminants pass through unhindered. Flow is then reversed, the adsorbent particles quickly settle and target proteins are desorbed by an elution buffer as in conventional packed bed chromatography. The eluate contains the target protein, at an increased concentration, free from particulates and ready from further chromatography purification.

The adsorbents are based on cross-linked agarose that has been modified through the inclusion of an inert, crystalline quartz, core material to provide the required high density for stable bed expansion.

STREAMLINE SP is a strong cation exchange adsorbent. The sulphonate groups maintain full protein binding capacity over the entire operating pH range. STREAMLINE DEAE is a weak anion exchanger, the number of ligand groups that are charged varies with pH (operating pH range 3-9).

Figure 4. STREAMLINE 25 Column for Method Development with Expanded Bed Absorption.

The stability of the expanded bed during feed application provides high binding capacities and minimal loss of product. This is in contrast to a fluidized bed where back-mixing results in much lower binding efficiency. Characteristically, the stable expanded bed behaves much closer to a packed bed in chromatography. Product yields from expanded bed adsorption with STREAMLINE are normally high and very efficient, in the range of 80-100%.

STREAMLINE DEAE and STREAMLINE SP have high chemical stability and can be used over wide pH ranges. Such high stability allows considerable flexibility when choosing conditions for adsorption and elution, as well as for efficient cleaning and sanitization.

STREAMLINE adsorbents also have high mechanical stability and can be expanded repeatedly with little generation of fines.

Determination of optimum pH and conductivity for expanded bed adsorption is often conveniently determined through breakthrough analysis of clarified material using the adsorbent in packed bed mode.

Efficient Cleaning-in-place (CIP) methods are used as an integral part of the complete process to maximize the life of the adsorbents and to minimize problems such as clogging. The cleaning protocol is dependent on the nature of the feed material and must be optimized on a case-to-case basis. They have enabled the repetitive use of the adsorbents, without affecting bed expansion and/or adsorption properties.

Task 7: Analysis of Mutant D. can. AFPs.

Antifreeze Capability Determinations of Mutant Dendroides Canadensis Antifreeze Proteins.

Recrysallization Inhibition Properties.
Recrystallization inhibition properties of potential antifreeze agents will be determined using splat-cooling method, by Dr. Wierzbicki's research group. Briefly, 10 microliters of the antifreeze solutions sample was drawn into a micropipette and splatted on the aluminum surface at the temperature of dry ice from a height of about 180 cm and allowed to anneal at the temperature of-8.1 °C for 120 minutes. During that time the crystal growth rate is measured using the polarized light microscope-video/printer system (Nikon model # DXC107 and Sony Video Mavigraph). This method has been widely accepted as a method of initial screening for recrystallization inhibition exhibited by all known non-equilibrium antifreeze agents. This procedure was performed for the antifreeze solution containing 1.0 mg/ml of NaCI. Addition of NaCI prevents the ice grain boundary from freezing, allowing the AFP molecules to reorient in solution and bind to ice. It has been shown that this is a necessary step to exclude the possibility that recrystallization effect is induced by high molecular weight particles that interfere with the grain boundary migration, not necessarily implying the stereospecific binding to ice needed for non-equilibrium antifreeze activity.

Nanoliter Osmometer Single Crystal Growth Experiments.
Determination if antifreeze solutions act as true non-equilibrium antifreeze protein by lowering the freezing point by Kelvin effect was done by observing the effect of its solution on the ice crystals growth morphology using Chifton Nanoliter Osmometer. This highly reproducible technique is utilized to determine if antifreeze molecules stereospecifically bind to ice, which is indicated by development of crystal facets at the water/ice boundary. It has to be emphasized here that showing the effect of potential antifreeze on crystal morphology by this technique (or alternatively by growth of single crystal of ice from solution) is essential in determination if antifreeze materials acts as the non-equilibrium (Kelvin effect) antifreezes. The same instrument Will be used to determine the freezing point depression hysteresis. The Clifton Nanoliter Osmometer is capable of obtaining such precise temperature settings that single ice crystals can be manipulated to have freezing and melting occurring at a DT corresponding to 1 mOsmos. Thus the freezing/melting process can be captured with relative ease.

Task 8: Environmental Fate Testing.

Toxicology & Environmental Fate Testing of the Mutated *D. Can.* Anti-Freeze Proteins.
All of the research to date on the antifreeze proteins that have been discovered, has shown that none of them have any biological toxicity including the D. can. antifreeze protein. Molecular modeling comparison of its structure to several know toxins has shown no structural similarities including active site comparisons and receptor binding domain comparisons. Also, no allergic responses to any of the antifreeze proteins have been published. Since these antifreeze molecules are naturally occuring proteins, they will rapidly degrade into non-toxic monomers, amino acids, which are already present in the environment.

A key driver in the development of advanced deicing formulations is the negative environmental impact of current materials such as propylene glycol and urea. Specifically, the most significant problem facing the use and application of these materials is their BOD. For example, in the case of propylene glycol, the toxicity of this material is extremely low, as is witnessed by the fact that it is widely used as a food additive. However, the BOD of this and other substitutes that have been considered to date are to high. If propylene glycol, urea, ethylene glycol, or any other type of organic matter is untreated when discharged into a body of water, the bacteria in the water will proceed to decompose it. In the process of decomposing the material, the bacteria will remove the dissolved oxygen from the water.

bacteria

Organic Matter + O_2 --------- CO_2 + waste products + NH_3 + energy + more bacteria

As noted earlier, current formulations such as those based on ethylene glycol biodegrade rapidly and cause a high or complete depletion of oxygen in the 5 day BOD test. These high BOD values lead to rapid depletion of oxygen in the waterways surrounding airfields. In the 5 day test a sample of the material in question is placed in water with a bacterial culture. Next the sample is incubated for five days, and after incubation, the change in the dissolved oxygen concentration is measured. Also during the 5 day period for the BOD test, 60 to 70 % of the ultimate oxygen demand is recorded.

As part of this task Aspen Systems will perform in-house testing and collaborate with Scientific Materials International Inc. laboratories to produce a statement of Ecological Behavior for each of the mutant D. can. antifreeze proteins that are produced during the course of this program.

Scientific Materials International Inc. laboratories, an independent testing organization, will perform the various environmental impact tests. Scientific Materials International located in Miami FL, is recognized as one of the most knowledgeable and respected environmental testing facilities and is routinely contracted by the Departments of Transportation and Defense to perform enviromental tests on new materials. The tests to be performed are in accordance with Aerospace Materials Specifications (AMS) 1424B and 1428B for aircraft anti-icing fluids and follow EPA approved protocols outlined in the Code of Federal Regulations handbook.

Environmental Fate and Toxicology Testing of the Mutated *D. can.* Antifreeze Proteins.
Working with Scientific Materials International Inc., EnviroSystems Inc., and BioPhysical Assay Laboratory LLC., Aspen Systems has three panels of tests to determine the environmental impact of the mutated Dendroides canadensis antifreeze proteins. The corresponding tests of these panels are listed below.

In addition to the Standard AMS Toxicity Tests, Aspen Systems Inc., in collaboration with EnviroSystems Inc. and the Biophysical Assay Laboratory LLC. have developed an environmental toxicology and fate analysis system composed of the three panels of assay tests determine the environmental impact of the mutated Dendroides canadensis antifreeze proteins.

AMS STANDARD Toxicity Tests

BOD
COD
Theoretical Total Oxygen Demand
5 Day Biodegradation Test
Trace Contaminants

TOXICOLOGY TESTING

PLANT TOXICITY TESTS

Seed Development Assay
Seed Germination Assay

Method: Seed Germination, Protocols for Short Term Toxicity Screening of Hazardous Waste Sites EPA/600/3-88/029 and Standard Practice for Conducting Early Seedling Growth, ASTM Designation: E 1598 - 94.

Test Species: Grasses
Exposure Period: 120 hours.
Control Acceptability: ≥90% germination.
Replicates: 3 to 4 replicates with 40 seeds per replicate.
Treatments: Minimum of 5 dilutions or specified dose.
Test Mode: Static non renewal.
Effect Measured: Germination.

Plant Development Assay

Root Tip Elongation Assay

Method: Seed Root Elongation, Protocols for Short Term Toxicity Screening of Hazardous Waste Sites EPA/600/3-88/029 and Standard Practice for Conducting Early Seedling Growth, ASTM Designation: E 1598 - 94.
Test Species: Grasses
Exposure Period: 120 hours
Control Acceptability: ≥90% germination
Replicates: 3 to 4 replicates with 5 to 10 seeds per replicate.
Treatments: Minimum of 5 dilutions or specified dose.
Test Mode: Static non renewal.
Effect Measured: Root growth.

Plant Growth Assay

Plant Growth Assay Evaluation

Method: Standard Practice for Conducting Early Seedling Growth, ASTM Designation: E 1598 - 94.
Test Species: Grasses
Exposure Period: 28 days
Control Acceptability: ≥90% germination
Replicates: 3 to 4 replicates with 5 to 10 seeds per replicate or as necessary based on test species.
Treatments: Minimum of 5 dilutions or specified dose.
Test Mode: Static non renewal.
Effect Measured: Plant height and or biomass, uptake of target compound.

ANIMAL TOXICITY TESTS

Invertebrate

Earthworm, *Eisenia foetida*, Acute Exposure Assays

Method: Protocols for Short Term Toxicity Screening of Hazardous Waste Sites EPA/600/3-88/029.
Exposure Period: 14 days
Control Acceptability: ≥90% survival
Age of Test Organism: Mature worms with well developed clitellum.
Replicates: 3 to 4 replicates with 10 worms per replicate.
Treatments: Minimum of 5 dilutions or specified dose.
Test Mode: Static non renewal.
Effect Measured: Survival

Daphnid Chronic and Modified Acute Exposure Assay (Invertebrate)

Method:	Daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test, Method 1002.0. Short-term Methods for Assessing the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms. Third Edition. EPA/600/4-91/002.
Exposure Period:	7 days.
Control Acceptability:	≤20% mortality, average of 15 juveniles produced per adult.
Age of Test Organism:	<24 hours.
Replicates:	10 per treatment.
Treatments:	Minimum of 5 dilutions.
Test Mode:	Daily renewal
Effect Measured:	Survival, reproduction success.

Vertebrate

Minnow Chronic and Modified Acute Exposure Assay (Vertebrate)

Method:	Flathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test, Method 1000.0. Short-term Methods for Assessing the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms. Third Edition. EPA/600/4-91/002.
Exposure Period:	7 days.
Control Acceptability:	≤20% mortality, mean weight of 0.25 mg/larvae
Age of Test Organism:	<24 hours.
Replicates:	10 per treatment.
Treatments:	Minimum of 5 dilutions.
Test Mode:	Daily renewal
Effect Measured:	Survival, growth.

Teratogenesis Assay

FETAX Assay (Vertebrate)

Method:	Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - *Xenopus*, ASTM Designation: E 1439-91.
Exposure Period:	96 hours
Control Acceptability:	≤10% mortality and or □10% abnormal development, □90% of larvae must reach stage 46 after 96 hours exposure.
Age of Test Organism:	Stage 8 to Stage 11.
Replicates:	Treatments - 2 replicates with 25 eggs per replicate; Control - 4 replicates with 25 eggs per replicate.
Treatments:	Minimum of 5 dilutions.
Test Mode:	Daily renewal
Effect Measured:	Survival, development, and developmental abnormalities

ENVIRONMENTAL FATE TESTING

Protein Breakdown Analysis

Amino Acid Detection

Breakdown products of the AFPs will analyzed by HPLC separation of AFPs with multiple transition element tags. Detection and quantitation of the amino acids is by newtron irradiation and radioactivity counting.

Nitrate/Nitrite Accumulation

Colorametric chemical analysis done according to;

EPA Protocol 353.2 and ASTM 4500-F for nitrates,

EPA Protocol 354.1 and ASTM 4500-B for nitrites.

BIOACCUMULATION ASSAYS

Plant Assay

Plant Bioaccumulation Evaluation

Method: Standard Practice for Conducting Early Seedling Growth, ASTM Designation: E 1598 - 94.

Test Species: At the discretion of client.

Exposure Period: 28 days

Control Acceptability: ≥90% germination

Replicates: 3 to 4 replicates with 5 to 10 seeds per replicate or as necessary based on test species.

Treatments: Minimum of 5 dilutions or specified dose.

Test Mode: Static non renewal.

Effect Measured: Plant height and or biomass, uptake of target compound.

Animal Assays

Invertebrate

Earthworm, *Eisenia foetida*, Bioaccumulation Evaluation

Method: Protocols for Short Term Toxicity Screening of Hazardous Waste Sites EPA/600/3-88/029 and Standard Guide for Conducting a Laboratory Soil Toxicity Test with the Lumbricid Earthworm, *Eisenia foetida* AST Designation: E 1676 - 95.

Exposure Period: 28 days

Control Acceptability: ≥90% survival

Replicates: 3-4 replicates with sufficient worms per replicate to provide required tissue biomass necessary for chemical analysis

Treatments: Specified Dose

Test Mode: Static non renewal.

Effect Measured: Survival, uptake of target compound.

Bivalve Bioaccumulation Evaluation

Method: Standard Guide for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks. ASTM Designation E 1022 - 94.

Exposure Period: 28 days

Control Acceptability: ≥90% survival

Replicates: 4 replicates with sufficient organisms per replicate to provide required tissue biomass necessary for chemical analysis

Treatments: Specified Dose

Test Mode: Static renewal.

Effect Measured: Uptake of target compound.

Daphnid Chronic and Modified Acute Exposure Assay

Method: Daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test, Method 1002.0. Short-term Methods for Assessing the Chronic Toxicity of Effluents

and Receiving Water To Freshwater Organisms. Third Edition. EPA/600/4-91/002.

Exposure Period: 7 days.
Control Acceptability: ≤20% mortality, average of 15 juveniles produced per adult.
Age of Test Organism: <24 hours.
Replicates: 10 per treatment.
Treatments: Minimum of 5 dilutions.
Test Mode: Daily renewal
Effect Measured: Survival, reproduction success.

Vertebrate

Minnow Chronic and Modified Acute Exposure Assay

Method: Flathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test, Method 1000.0. Short-term Methods for Assessing the Chronic Toxicity of Effluents and Receiving Water To Freshwater Organisms. Third Edition. EPA/600/4-91/002.
Exposure Period: 7 days.
Control Acceptability: ≤20% mortality, mean weight of 0.25 mg/larvae
Age of Test Organism: <24 hours.
Replicates: 10 per treatment.
Treatments: Minimum of 5 dilutions.
Test Mode: Daily renewal
Effect Measured: Survival, growth.

Fish Bioaccumulation Evaluation

Method: Standard Guide for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks. ASTM Designation E 1022 - 94.
Exposure Period: 28 days
Control Acceptability: ≥90% survival
Replicates: 4 replicates with sufficient organisms per replicate to provide required tissue biomass necessary for chemical analysis
Treatments: Specified Dose
Test Mode: Static renewal.
Effect Measured: Uptake of target compound.

GROWTH STIMULATION ASSAY

Microbial

Microtox® Assay

Method: Standard Test Method for Assessing the Microbial Detoxification of a Chemically Contaminated Water and Soil Using a Toxicity Tests with a Luminescent Marine Bacterium ASTM Designation: D 5660-96 plus Microbics Corporation Microtox® manual.
Exposure Period: 15 to 30 minutes.
Control Acceptability: NA
Replicates: Duplicate.
Treatments: Up to 13 dilutions.
Test Mode: Static non renewal.
Effect Measured: Effect of compound on survival of bacteria.

Task 9. Scale-up Expression and Purification of the Mutated *D. can.* Antifreeze Protein.

Dr. Patrick Lucy of the Collaborative BioAlliance Inc. have has been contacted by Aspen Systems to discuss the development of a large scale fermentation process for the production of the *D. can.* antifreeze protein. Dr.. Lucy has experience in the development and scale-up of fermentation processes including the *Pichia pastoris* yeast expression system as currently being developed in Aspen System's laboratories. Dr. Lucy has consulted in the design and implementation of fermentation units for various pharmaceutical abd biotechnology companies, including large scale processes of up to 200,000 L. Collaborative BioAlliance will also perform an economic analysis of the scale-up and full scale production of the antifreeze protein. These estimates must await the results of testing on the purified protein to determine if our expressed protein works at as low concentrations as the native protein. Economic feasibility studies would hinge on the concentration of protein required to elicit its deicing effect. All indications of its ice crystal formation inhibition capabilities suggest low protein concentrations will be required for this purpose.

Task 10. Formulation of multicomponent anti-icing fluids to AMS standards.

Using Aerospace Material Specifications (AMS) 1424 (SAE Type I "Newtonian") and AMS 1428 (SAE Type II "Non-Newtonian") as property and qualification templates, this task will focus formulation of multicomponent fluids to meet the specifications, while utilizing the newly developed AFPs. Due to the complex requirements placed on deicing fluids, it will be necessary to formulate a fluid mixture with colorants, and possibly additives such as anti-corrosive and thickeners. The application of antifreeze proteins will allow us to formulate anti-icing formulations that is inherently environmentally more compatible than standard formulations.

Task 11. Testing of multicomponent fluids for anti-icing efficacy with in field testing.

This task will focus on the qualification and field testing of the fluids formulated in Task 5. The bulk of the testing will be designed to qualify the AAP compounds as AMS 1424 (SAE Type I) and AMS 1428 (SAE Type II) fluids and pertinent Federal Aviation Administration (FAA), Industry, Military, and EPA requirements. **Table 3.** summarizes the testing and qualification plan for fluid formulations based on the mutant antifreeze protein.

Table 3. Summary of testing and qualification plan for fluid formulations based on the *D. can.* antifreeze proteins.

TESTING REQUIREMENTS	TEST METHOD
TECHNICAL TESTS	
Runway Deicing Effectiveness	AMS
Runway Friction Testing	AMS
Material Composition	AMS
Material Appearance	AMS
FLUID STABILITY	
Storage Stability	ASTM F 1105
Thermal Stability	ASTM E 70
Thin Film, Thermal Stability	AMS
Exposure to Dry Air Stability	AMS
Shear Stability	AMS
Hard Water Stability	AMS

EFFECT ON AIRCRAFT MATERIALS	
Sandwich Corrosion	ASTM F 1110
Total Immersion Corrosion	ASTM F 483
Low-Embrittling Cadmium Plate	ASTM F 1111
Stress-Corrosion Resistance	ASTM F 945
Hydrogen Embrittlement	ASTM F 519
EFFECT ON TRANSPARENT PLASTICS	
Stretched Acrylic Plastic	ASTM F 484
Polycarbonate Plastic	ASTM F 484
EFFECT ON PAINTED SURFACES	ASTM F 502
EFFECT ON UNPAINTED SURFACES	ASTM F 485
PAVEMENT SCALING RESISTANCE	ASTM C 672
ANTI-ICING PERFORMANCE	
Water Spray Endurance Test	ASTM D 1193
High Humidity Endurance Test	ASTM D 1193
Aerodynamic Acceptance Test	AMS

Aspen Systems will team with the University of Quebec at Chicoutimi, GRIEA Research Group to complete Holdover Time Qualification and Aerodynamic Qualification Tests.

MILESTONES FOR THESE TASKS ARE:

FY1999 Milestones:	Planned Completion;	Completed Date;
1.0 DNA Oligonucleotide Synthesis	06/1998,	06/1998
2.0 Gene mutation & bacterial cloning	09/1999,	09/1999
3.0 DNA Sequence Analysis	10/1999,	10/1999
4.0 Yeast Cloning, Selection & Analysis	12/1999,	01/2000
FY2000 Milestones:		
5.0 Mutant D. can. Antifreeze Protein Expression	04/2000,	05/2000
6.0 Mutant D. can. Antifreeze Protein Purification	04/2000,	05/2000
7.0 Antifreeze capabilities determinations of mutant D. can. AFPs	04/2000	09/2000
8.0 BOD, Toxicity, Environmental Fate testing of the mutant D. can. AFPs	04/2000,	06/2000
FY2001 Milestones:		
9.0 Scale-up production of the mutant D. can. AFPs	04/2001	On Going
Contract Bioprocess Center for Scale-up Expression	07/2000	08/2000
Bioprocess Center Scale-up Protein Expression	04/2001	On Going
Bioprocess Ctr. Purification Scheme Analysis	04/2001	On Going
FY2002 Milestones:		
10.0 Multicomponent Anti-icing Fluid Formulation	04/2002	
Fluid Viscosity Formulation	10/2001	
Anti-icing Fluid Activity Testing	10/2001	
Protein Stability Testing	01/2002	
11.0 Anti-Icing Fluid Field Testing	04/2002	
Contract Univ. Quebec for Aerodynamic Testing	06/2001	

Materials Compatibility Testing	04/2002
Anti-Icing Fluid Activity Testing	04/2002

ACCOMPLISHMENTS:

Summary

During the first year of the SERDP Pollution Prevention Program #1110, Aspen Systems Inc. has accomplished all of the program tasks on or ahead of their time points. These accomplishments to date include the amino acid sequence analysis and binding domain comparisons of the *Dendriodes canadensis* antifreeze protein with other published AFP sequences. The design, synthesis and conformational sequencing of the mutagenesis DNA oligonucleotides to be used to mutate the *D. can.* AFP gene. The cloning of the *D. can.* AFP gene DNA into the pALTER II mutagenesis cloning vector. The confirmation of the mutated sequences by DNA sequencing. The cloning of the mutant *D. can.* AFPs genes into the yeast, Pichia pastoris, and their insertion confirmed by PCR analysis. These clones have since been proven to be expressing an immunoreactive protein that is secreted into the media which confirms the presence of an AFP.

The second year of this program has been seen the continued development of the antifreeze proteins expression and purification protocols. The most important accomplishment at this stage of the program has been the completion of the environmental toxicology and fate testing that was done by EnviroSystems Inc. of Portsmouth NH. We have also contract a bioprocess center to do the scale-up expression and purification of our proteins.

Task 5: Expression of the Mutated D. can. AFPs in *Pichia pastoris*:

Set-up of the New Brunswick 14 liter Fermentor:

The New Brunswick MF-114 fermentor has been installed and the necessary accessories purchased in preparation for the initial scale-up expression run of the *D. can.* gene transformed *Pichia* yeast. This included the retrofitting of the apparatus with an antifoam probe, a dissolved oxygen (D.O.) probe and monitor, and a pH probe, pH controller and two solvent pumps for acid and base addition to the reaction vessel. The water line and oxygen lines have also been connected and calibrated. A dry test run of the agitation, cooling and electronic systems was done to insure the readiness of the apparatus.

For further characterization of the expressed proteins can continue, larger sample needs to be expressed which can then be purified and analyzed for physical characterization and the freezing point depression capabilities of the proteins. A fermentation of the *Pichia* clones by growing them overnight in a flask containing BMGY media, (supplemented with glycerol as a carbon source). The yeast are then transferred to the fermentor and grown in media minus glycerol until a spike was measured by the dissolved oxygen (D.O.) probe. Methanol was then added as a carbon source and induction agent. The clones was then incubated for an additional 6 days with the addition of methanol every 12 hr. The fermentation media and cells were harvested and retained for purification of the STREAMLINE 25 column. This fermentation sample was processed by applying it to the STREAMLINE 25 SP column then eluting the sample with 0.5M NaCl as discussed below.

We continue developing a unique media for the fermentation of the *Pichia pastoris* yeast containing the mutated *Dendriodes canadanesis* antifreeze protein genes. This new media is based on a

combination of the two different media that have been used in the past whose recipes were found described in the literature . This new media is designed to boost the nitrogen source for the production of the protein. Using this method we have completed another 10 liter fermentation of the *Pichia* clone, using the improved media and method found in the literature. The new protocol is as follows;

Baffled flasks containing a total of 5-10% of the initial fermentation volume of BMGY (30% of the total flask volume) were inoculated from a frozen glycerol stock of *Pichia* clone S6. The flasks were grown at 30°C, 250-300 rpm, 16-24 hours until OD_{600} = 2-6. To accurately measure OD_{600} > 1.0, a sample of the culture was diluted 10 fold before reading. The fermentor was sterilized with the Fermentation Basal Salts medium containing 4% glycerol.

After sterilization and cooling, the temperature was set to 30°C, agitation and aeration to operating conditions were set at 800rpms and 1.2 liters/min. respectively. The pH of the Fermentation Basal Salts medium was adjusted to 5.0 with 28% ammonium hydroxide. Then 4.35 ml PTH trace salts/liter of Fermentation Basal Salts medium were added aseptically.

We inoculated the fermentor with approximately 5-l0 to initial fermentation volume from the culture generated in the inoculum shake flasks. The D.O. was close to 100% before the culture starts to grow. As the culture grew, it consumed oxygen, causing the D.O. to decease. We were sure to keep the D.O. above 20% by adding oxygen as needed.

Grow the batch culture until the glycerol is completely consumed (18 to 24 hrs.). This is indicated by an increase in the D.O. to 100%. The length of time needed to consume all the glycerol will vary with the density of the initial inoculum.

Sampling is performed at the end of each fermentation stage and at least twice daily. We take 10 ml samples for each time point, then take 1 mL aliquots from this 10 mL sample. Samples are analyzed for cell growth (OD_{600} and wet cell weight), pH, microscopic purity and protein concentrations. These samples are freeze the cell pellets and supernatants at 4°C for later analysis.

Terminate glycerol feed and initiate induction by starting a 100% methanol feed containing 12 ml PTM trace salts per liter of methanol. Set the feed rate to 3.6 ml/hr per liter initial fermentation volume. During the first 2-3 hours, methanol will accumulate in the fermentor and the dissolved oxygen values will be erratic while the culture adapts to methanol. Eventually the D.O. reading will stabilize and remain constant.

If the D.O. cannot be maintained above 20%, stop the methanol feed, wait for the D.O. to spike and continue on with the current methanol feed rate increase agitation, aeration, pressure or oxygen feeding to maintain the D.O. above 20%.

When the culture is fully adapted to methanol utilization (24 hrs.), and is limited on methanol, it will have a steady D.O. reading and a fast D.O. spike time (generally under 1 minute). Maintain the lower methanol feed rate under limited conditions for at least 1 hour after adaptation before doubling the feed. The feed rate is then doubled to ~7.3 ml/hr/liter initial fermentation volume.

After 2 hrs. at the 7.3 ml/hr/liter feed rate, increase the methanol feed rate to ~10.9 ml/hr per liter initial fermentation volume. This feed rate is maintained through the remainder of the fermentation. The entire methanol fed batch phase lasts approximately 70 hours with a total of approximately 740 ml methanol fed per liter of initial volume. (However, this varies for different

proteins.) Our results demonstrate that we can enhance the protein yield produced by the *Pichia pastoris* by improving our fermentation conditions. And, that we can adapt our purification scheme to be optimized to the different media from which the antifreeze protein must be isolated.

Task 6: Mutant D. can. Protein Purification.

As reported previously, we have standardized our laboratory scale purification scheme and as quality control for the isolation of our proteins from the fermentation media. Following each column run, samples from the fermentation of a *Pichia* clone are routinely analyzed for purity by SDS-PAGE gels stained with Coomassie Blue.

In order to purify the mutated *Dendriodes canadensis* antifreeze proteins from the crude fermentation media and cells, we apply the fermentation media from each run directly onto the expanded bed absorption chromatography STREAMLINE SP column. After the column is washed 10X, the buffer flow was reversed. When the fermentation media that is loaded onto the column had a pH of 3.5, the first elution buffer used was 0.0M NaCl - pH 6.0. The *D. can.* proteins are then eluted with a 0.5 M NaCl - pH 6.0 buffer. We collected 40 - 15 mL fractions. We then stripped all of the remaining proteins off the column with a 1.0 M NaCl - pH 6.0 buffer, again collecting 40 - 15 mL fractions.

Task 7: Analysis of Mutant D. can. AFPs.

Recrystallization Inhibition Experiments

Recrystallization inhibition properties of potential antifreeze agents were determined using splat-cooling method as originally proposed by Knight (1988) and modified by Warren (1993) and our research group. Briefly, 10 microliters of the antifreeze solutions sample was drawn into a micropipette and splatted on the aluminum surface at the temperature of dry ice from a height of about 180 cm and allowed to anneal at the temperature of -8.1 °C for 120 minutes. During that time the crystal growth rate is measured using the polarized light microscope-video/printer system (Nikon model # DXC107 and Sony video mavigraph printer model # 11232). This method has been widely accepted as a method of initial screening for recrystallization inhibition exhibited by all known non-equilibrium antifreeze agents. This procedure was performed for the antifreeze solution containing 1.0 mg/ml of NaCl. Addition of NaCl prevents the ice grain boundary from freezing, allowing the AFP molecules to reorient in solution and bind to ice. It has been shown that this is a necessary step to exclude the possibility that recrystallization effect is induced by high molecular weight particles that interfere with the grain boundary migration, not necessarily implying the stereospecific binding to ice needed for non-equilibrium antifreeze activity (Knight 1994).

Nanoliter Osmometer Single Crystal Growth Experiments

Determination if antifreeze solutions act as true non-equilibrium antifreeze protein by lowering the freezing point by Kelvin effect was done by observing the effect of its solution on the ice crystals growth morphology using Chifton Nanoliter Osmometer (Chifton Technical Physics). This highly reproducible technique is utilized to determine if antifreeze molecules stereospecifically bind to ice, which is indicated by development of crystal facets at the water/ice boundary (Hon et al.). It has to be emphasized here that showing the effect of potential antifreeze on crystal morphology by this

technique (or alternatively by growth of single crystal of ice from solution) is essential in determination if antifreeze materials acts as the non-equilibrium (Kelvin effect) antifreezes. Later the same instrument can be used to determine the freezing point depression hysteresis. Clifton nanoliter osmometer apparatus consists of two parts. The f1rst being the control unit which is used to directly control the temperature of the second part, the thermal stage. The Clifton Nanoliter Osmometer is capable of obtaining such precise temperature settings that single ice crystals can be manipulated to have freezing and melting occurring at a delta-T corresponding to 1 mOsmos. Thus the freezing/melting process can be captured with relative ease.

An eight ring sample holder was immersed into "type B oil. " This oil having a density greater than that of the sample is used to keep the sample in place in the holder. Upon addition of this oil approximately 10 nanoliter of sample was drawn into a pipette that was made from a 10,ul pipette that was drawn over a Bunsen burner to accommodate this small volume. The sample was then displaced from the capillary into the sample holder. Immediately after delivering the sample a "type A oil" was added to further ensure sample stabilization. This type A oil has a density less than that of the sample and type B oil. Therefore, the sample was isolated in a hydrophobic environment which allowed for accurate study of crystal growth

Completing the loading process, the sample was "flash" frozen to a temperature approximately equal to -40 °C. After this "deep" freezing has occurred the samples were slowly melted until the point at which one crystal remained. At this point, the temperature was fined tuned and a picture of the morphological characteristics of each individual crystal was obtained and compared to that of AFP-I and deionized water.

Due to limited amounts of the samples, we conducted nanoliter osmometer testing alone, which provided us with sufficient amount of information to determine the potential for their non-equilibrium antifreeze activity.

Results: Report from Dr. Andrea Weirzbicki, Univ. So. Alabama.

Ten samples were analyzed using nanoliter osmometer. During testing all samples showed similar behavior as far as ice crystal growth inhibition is concerned, therefore this summary will apply to all samples tested. All samples showed ability to modify ice crystal morphology resulting in, depending on the growth rate, flat hexagonal tablets or short truncated bipyramids. This behavior indicates specific interactions with ice, which is a necessary for developing a non-equilibrium freezing point depression via the Kelvin effect. Inactive proteins/polypeptides do not affect ice crystals morphology, yielding spherical shapes of ice crystal (similar to control crystals grown from pure water). You can clearly observe that the effect of these antifreezes on ice morphology changes with its concentration: from regular bipyramidal (high concentration) through truncated bipyramids to flat hexagonal tablets (low concentration). For the bipyramidal morphology non-equilibrium freezing point depression can be determined, whereas for the hexagonal tablets morphology the value of freezing point depression is so close to zero that it can not be determined. There is not enough of protein to limit the growth of ice crystal, similarly we could not determine the freezing point depression for the samples analyzed due to insufficient concentration of the samples.

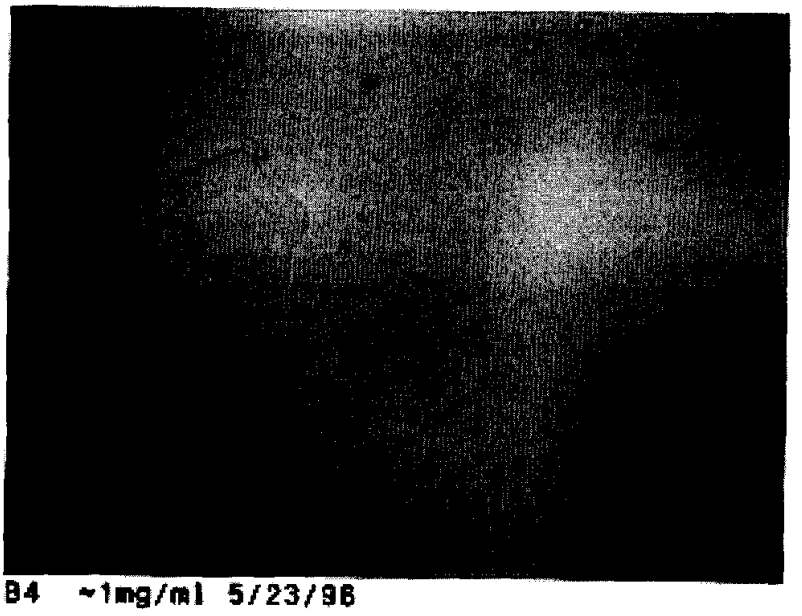


Figure 38: Nanoliter Osmometery of Aspen's B4 Clone

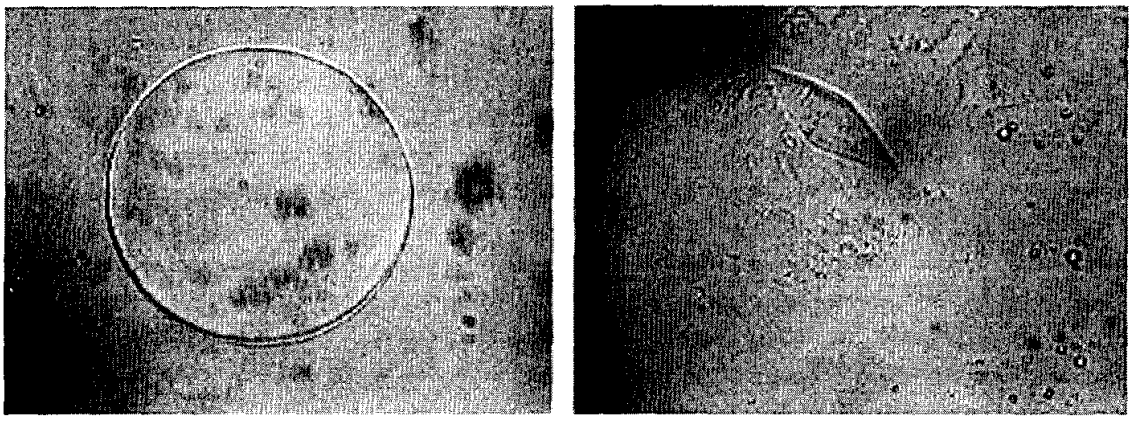


Figure 39: Nanoliter Osmometery of H_2O and the Mutated *D. can.* 11 Clone

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Hon W.C, Griffith M., Chong P., Yang D.S.C. (1994) Extraction and Isolation of Antifreeze Proteins from winter Rye (Secale cereale L.) Leaves. Plant Physiology 104: 971-980.

Task 8: Environmental Fate Testing.

Acute & Chronic Toxicity Assay Testing Of Antifreeze Proteins.

The Aspen Systems antifreeze proteins were evaluated for their acute and chronic environmental toxicity using freshwater species, *Ceriodaphnia dubia* and *Pimephales promelas*. Toxicity was also evaluated using the lettuce seed, *Lactuca sativa*, the frog, *Xenopus laevis*, and Microtox analysis using the eutaurine bacteria, *Vibrio fischeri*.

SPECIES / ENDPOINT	EXPOSURE	LC-50	EC-50	NOEC
Ceriodaphnia dubia				
Survival	48 Hours	>10.0 mg/L		>10.0 mg/L
	7 Days	>10.0 mg/L		>10.0 mg/L
Reproduction	7 Days	>10.0 mg/L		
Pimephales promelas				
Survival	48 Hours	>10.0 mg/L		>10.0 mg/L
	7 Days	>10.0 mg/L		>10.0 mg/L
Growth	7 Days			1.25 mg/L
Lactuca sativa				
Germination	120 Hours	>10.0 mg/L		>10.0 mg/L
Growth	120 Hours	>10.0 mg/L		>10.0 mg/L
Xenopus laevis				
Survival	96 Hours		>10.0 mg/L	>10.0 mg/L
Development	96 Hours		>10.0 mg/L	>10.0 mg/L
Growth	96 Hours		>10.0 mg/L	5.0 mg/L
MICROTOX				
Survival	5 Minutes		>10.0 mg/L	
Survival	15 Minutes		>10.0 mg/L	

The samples provided by Aspen Systems Incorporated did not exhibit any signs of acute toxicity to the daphnid, *Ceriodaphnia dubia*, or the minnow, *Pimephales promelas*, during the initial 48 hour exposure period. The sample had an observed sublethal effect on minnow growth, but not on daphnid reproduction, during the 7 day exposure period. The sample had an effect on tadpole growth but not on tadpole survival or development. There was no observed effect on the survival or growth of the lettuce, *L. sativa*, or the performance of the marine bacteria, *V. fischeri*, in the Microtox analysis.

ACUTE & CHRONIC TOXICITY ASSAY RESULTS

Daphnid Acute/Chronic Survival -No Effect Concentration = > 10 mg/L

	Reproduction -No Effect Concentration = > 10 mg/L
Minnow Acute/Chronic	Survival -No Effect Concentration = > 10 mg/L Growth -No Effect Concentration = 1.25 mg/L
Earthworm Acute	Survival -No Effect Concentration = > 10 mg/L Reproduction -No Effect Concentration = > 10 mg/L
FETAX	Teratogenicity -No Effect Concentration = > 10 mg/L Growth -No Effect Concentration = > 10 mg/L
Seed Germination	No Effect Concentration = > 10 mg/L
Seed Root Tip Growth	Growth -No Effect Concentration = > 10 mg/L
MicroTox Analysis	MicroTox EC-50 = > 10 mg/L

Aspen Systems' natural, biodegradable anti-icing antifreeze proteins have been proven to be non-toxic and environmentally benign, making them excellent replacements for propylene/ethylene glycols as anti-icing agents in aircraft anti-icing fluids.

TOXICOLOGICAL EVALUATION OF A DE-ICING COMPOUND: AUGUST 2000

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August 2000
Reference Number ASPEN8982-00-08

STUDY NUMBER 8982
EXECUTIVE SUMMARY

The following summarizes the results of bioassays conducted on product S1-56 received during August 2000. Samples were provided by Aspen Systems Incorporated, Marlborough, Massachusetts. Acute and chronic toxicity was evaluated using the freshwater species, *Ceriodaphnia dubia* and *Pimephales promelas.* Toxicity was also evaluated using the lettuce seed, *Lactuca sativa,* the frog, *Xenopus laevis,* and Microtox@ analysis using the estuarine bacteria, *Vibno fischeri.*

Species / Endpoint	Exposure	LC-50	EC-50	NOEC
Ceriodaphnia dubia				
Survival	48 Hours	>10.0 mg/L		>10.0 mg/L
	7 Days	>10.0 mg/L		>10.0 mg/L
Reproduction	7 Days			>10.0 mg/L
Pimephales promelas				
Survival	48 Hours	>10.0 mg/L		>10.0 mg/L
	7 Days	>10.0 mg/L		>10.0 mg/L
Growth	7 Days			1.25 mg/L
Lactuca sativa				
Germination	120 Hours		>10.0 mg/L	>10.0 mg/L
Growth	120 Hours		>10.0 mg/L	>10.0 mg/L
Xenopus laevis				
Survival	96 Hours		>10.0 mg/L	>10.0 mg/L
Development	96 Hours		>10.0 mg/L	>10.0 mg/L
Growth	96 Hours		>10.0 mg/L	5.0 mg/L
Microtox©				
Survival	5 Minutes		>10.0 mg/L	
Survival	15 Minutes		>10.0 mg/L	

TOXICOLOGICAL EVALUATION
OF A DE-ICING COMPOUND:
AUGUST 2000

Aspen Systems Incorporated
Marlborough, Massachusetts

1.0 INTRODUCTION

Acute toxicity tests involve preparing a series of test concentrations by diluting sample with control water. Groups of test animals are exposed to each test concentration and a control for a specified period. The mortality data for each concentration can be used to calculate (by regression) the median lethal concentration or LC-50, defined as the concentration of sample which kills half of the test animals. The EC-50, or the concentration that affects half of the test organisms, can also be calculated in this manner. Samples with a high LC-50 or EC-0 value are less likely to cause significant environmental impacts. The data can also be analyzed to determine the no effect level. This Acute No Observed Effect Concentration (A-NOEC) is defined as the highest tested sample concentration which causes no significant mortality.

Chronic toxicity tests measure sublethal effects, exposing test organisms to sample samples during a sensitive period in the life cycle. Minnow chronic tests measure survival and growth (weight) during the first seven days post hatch; daphnid chronic tests measure juvenile production. Using Analysis of Variance techniques to evaluate the data, it is possible to determine the lowest tested concentration that had an effect (LOEC) and the highest tested concentration where no effect (C-NOEL) was observed. The geometric mean of these points is the maximum allowable toxicant concentration (MATC) or chronic value.

This report presents the results of toxicity tests on a de-icing compound provided by Aspen Systems Incorporated, Marlborough, Massachusetts. Testing was based on programs and protocols developed by the US EPA (1994) and involved conducting chronic toxicity tests with the freshwater species, *Ceriodaphnia dubia* and *Pimephales promelas.* Toxicity was also evaluated using the lettuce seed, *Lactuca sativa,* the frog, *Xenopus laevis,* and Microtox© analysis. Testing was performed at EnviroSystems, Incorporated (ESI), Hampton, New Hampshire.

2.0 MATERIALS AND METHODS

2.1 General Methods

Toxicological and analytical protocols used in this program follow procedures outlined in *Methods forMeasuring the Acute Toxicity of Samples to Freshwater and Marine Organisms* (EPA 1993), Short *Term Methods for Estimating Chronic Toxicity of Samples and Receiving Waters to Freshwater Organisms* (EPA 1994), *Standard Methods for the Examination of Water and* Wastewater(APHA 1995), *Attachment G: NPDES Toxicity Testing, Monitoring and Reporting Tips and Common Pitfalls* (EPA 1999) and *Aquatic Toxicology and Risk Assessment: Volume 11.05* (ASTM 1996). These programs provide standard approaches for the evaluation of chronic toxicological effects of discharges on aquatic organisms. and for the analysis of water samples.

2.2 Test Species

C. dubia, cultured at ESI, were maintained in synthetic, soft reconstituted water at 25±1 °C with a photoperiod of 16:8 hours light:dark. Cultures are fed daily with a yeast/trout chow/Cerophyll (YTC) mixture supplemented with Selenastrum capricomutum (algae) (EPA 1994). Adults on a brood board were isolated 24 hours prior to test start and allowed to reproduce overnight. Organisms used in the assay were <24 hours old juveniles from broods of >_8 juveniles.

P. promelas, <_24 hours old, were from cultures maintained by Cosper Environmental Services, Inc. Test organisms were transferred from acclimation tanks to test chambers using a large bore glass tube, minimizing the amount of water added to the test solution.

L.. safiva seeds were obtained from Carolina Biological Supply, Inc. Seeds selected were identified as a basic, wild type, stock. No lot numbers were assigned to the seeds by the supplier. Prior to use, seeds were placed in a sieve and graded to size.

Adult frogs, Xenopus laevis, used in the assays were purchased from a commercial supply source. At the laboratory, adult specimens were maintained in a synthetic moderately hard water re-circulating system. Temperature was maintained at 24±2°C and the photoperiod set at 12:12 light:dark. Frogs were fed a commercial diet.

Males and females were kept separate until testing began. For the Microtox(D evaluation, the luminescent marine bacteria, Vibrio fischeri, were supplied by the manufacturer in a lyophilized form which is stored in the freezer until it is rehydrated for the analysis.

2.3 Test Compound and Dilution Water

Two vials of test compound, identified as S1-56, were delivered to ESI by Aspen Systems Incorporated on August 23, 2000. Samples were stored at 4°C and warmed to 25±1 °C prior to preparing test solutions.

Dilution water for the daphnid and minnow assay was synthetic, soft reconstituted water prepared at ESI according to protocol (EPA 1994). This water has been used to successfully culture freshwater organisms since 1992.

Dilution water for the *L.* sativa assay was de-ionized water. Dilution water for the FETAX assay was mixed according to protocol (ASTM 1997). Dilution water for the Microtox@ assay was provided by the manufacturer, Azur Environmental.

Total residual chlorine (TRC) was measured by amperometric titration (MDL 0.05 mg/L) in the sample and diluent samples prior to use in the assays. Samples with >_0.05 mg/L TRC were dechlorinated using sodium thiosulfate (EPA 1994).

2.4 Bioassay Procedure and Statistical Analysis

2.4.1 Chronic Toxicity Tests - C. *dubia* and P. *promelas*

The 7 day chronic toxicity tests were conducted according to protocol (EPA 1994), which called for the daily renewal of test solutions. Test treatments were 10.0 mg/L, 5.0 mg/L, 2.5 mg/L, 1.25 mg/L, 0.625 mg/L, and 0.3125 mg/L sample with laboratory water diluent control. Dissolved oxygen, pH, and conductivity were measured in one replicate of each new test solution. Fresh stock solutions of test sample S1-56 were prepared daily.

Test chambers for the daphnid assay were 30 mL portion cups containing 15 mL of test solution in each of 10 replicates with 1 organism/replicate. Survival and juvenile production were monitored daily. Daphnids were fed 100 yL each of YTC and algae after daily renewals.

Test chambers for the fathead minnow assay were 600 mL beakers with 250 mL of solution in each of 4 replicates containing 10 organisms/replicate. Prior to daily minnow renewals, survival and dissolved oxygen were measured in all replicates, and pH and conductivity were measured in one replicate of each concentration. Fish were fed newly hatched *Artemia* nauplii daily. Dead nauplii from previous feedings were removed during daily renewals. On Day 7 of the assay, surviving fish were rinsed in deionized water and tranquilized using FinquelO tricaine methanesulfonate. Fish were placed on tared weighing pans and dried overnight at **104°C** to obtain dry weights to 0.01 mg. Final dry weight/fish for statistical comparison was calculated by dividing the net dry weight by the number of organisms introduced at the initiation of the assay.

When applicable, survival data from the initial 48 hour exposure period were analyzed for acute toxicity using a program developed by Stephan (1982) which computes LC-50 values using the Spearman-Karber and Probit methods. If survival in the highest test concentration was >50 mg/L, the LC-50 value was determined by direct observation of the raw data. The A-NOEC was the highest test concentration which caused no significant mortality.

C. dubia reproduction and P. promelas growth data, normal and homogenous, were analyzed using Dunnett's Test (EPA 1994). Replicate data were combined to determine the statistical significance of any differences between the treatments and controls. Statistical significance was accepted at « <0.05.

2.4.2 Germination and Root Tip Elongation Assay - L. *sativa*

The 120 hour seed germination and root tip elongation assay was conducted at 24±2°C in total darkness with 4 replicates per concentration. Test treatments were 10.0 mg/L, 5.0 mg/L, 2.5 mg/L, 1.25 mg/L, 0.625 mg/L, and 0.3125 mg/L sample with laboratory water diluent control. Test chambers were 100 x 15 mm petri dishes with absorbent pads. Test solutions were added to each test chamber to saturate the pad before adding 5 seeds per chamber. A t the end of the 120 hour exposure period, germination data was collected and each root tip was measured from tip to seed.

Root tip data, normal and homogenous, were analyzed using the Bonferroni t-Test to determine the highest test concentration which caused no significant reduction in growth, also referred to as the NOEC.

2.4.3 FETAX Assay - X. laevis

The 96 hour FETAX screening assays were conducted in a static renewal test mode. Approximately 50% of the test solution was replaced every 24 hours. Test treatments were 10.0 mg/L, 5.0 mg/L, 2.5 mg/L, 1.25 mg/L, 0.625 mg/L, and 0.3125 mg/L sample with FETAX solution diluent control. The assay was started with the addition of fertilized eggs between developmental stages 8 and 11 to the test solution. The experimental design specified two replicates with 25 eggs per replicate. Test chambers were 60 ml petri dishes and contained 25 ml of test solution. Temperatures were maintained at 24+2°C and the photoperiod was set at 12 hours light and 12 hours dark. The control treatment was identical to the test treatments and consisted of the FETAX solution. At the end of the assay, the number of surviving embryos was determined. In addition, the number of malformed embryos (tadpoles) was also determined. Malformations were based on the Atlas of Abnormalities (Nieuwkoop and Faber, 1975).

Adult spawning was induced by a 500 IU injection of human chorionic gonadotropin into the dorsal lymph sac. Paired adults were placed in spawning chambers in the FETAX solution at a temperature of 24°C without light. Fertilized eggs were collected and inspected to determine general acceptability. Eggs from adult pairs were kept separate and not mixed together. Prior to use in the assay, eggs were exposed to a 2% solution of pH adjusted L-cysteine to remove the outer jelly coat. After removal of the jelly coat, eggs were inspected and sorted based on their developmental stages. Eggs selected for use in the assay were between developmental stage 8 and 11.

Survival, growth and developmental data were evaluated using TOXSTATO software to determine potential significant differences between the treatments and controls. TOXSTAT© software was also used to determine LC-50 and EC-50 values (EPA 1994).

2.4.4 Microtox® Evaluation - V. fischeri

MicroTox©testing followed protocol developed by the instrument's manufacturer. Analysis was conducted using the "100% Test" protocol and EC-50 values were determined after 5 and 15 minutes of exposure. The test used double sets of controls and two replicates/concentration. Test concentrations were 10.0 mg/L, 5.0 mg/L, 2.5 mg/L, 1.25 mg/L, 0.625 mg/L, and 0.3125 mg/L sample diluted with diluent supplied by the manufacturer. Samples were salinity adjusted to approximately 20 ppt using solid sodium chloride prior to analysis.

This test compares the amount of light emitted by the bacteria after a specified exposure period against the amount emitted by a diluent only control. Negative impacts are associated with a reduction in light output by the bacteria during the exposure period. The data is used to determine the median effected concentration, EC-50. The EC-50 is defined as the concentration
which causes a 50% reduction in light emission as compared to the control.

2.5 Quality Control

As part of the laboratory quality control program, reference toxicant evaluations are conducted on a regular basis for each test species. These results provide relative health and response data while allowing for comparison with historic data sets. The Sodium Dodecyl Sulfate reference toxicant assay conducted on September 27, 2000 resulted in a 48 hour LC-50 value of 26.4 mg/L (Binomial Method) for C. *dubia.* The sodium dodecyl sulfate reference toxicant assay conducted during September 2000 resulted in a 48 hour LC-50 value of 27.0 for P. *promelas.* These values were within two and one standard deviations of the historic mean for each species, respectively.

The 96 hour *L. sativa* reference toxicant assay conducted on October 9, 2000 resulted in an IC-25 value of 0.77 mg/L Cadmium and a NOEC value of 0.10 mg/L Cadmium. These values were within one standard deviation of the historic laboratory mean.

The X. *laevis* reference toxicant assay conducted on September 13, 2000 resulted in a 96 hour EC-50 value of 7.97 mg/L (Spearman-Karber Method). This value was within one standard deviation of the historic laboratory mean. Survival in the 2500 mg/L 6-aminonicotinamide treatment was 58% while normal development in the 5.5 mg/L 6-aminonicotinamide solution was 54%.

The Microtox@ reference toxicant assays conducted on December 14, 1999 resulted in a 5 minute EC-50 value of 16.2 mg/L phenol and a 15 minute EC-50 value of 3.8 mg/L zinc sulfate. Test acceptability ranges are 13-26 mg/L phenol and 3-10 mg/L zinc sulfate.

3.0 RESULTS AND DISCUSSION

Results of the chronic and modified acute exposure assays conducted using C. *dubia* and P. promelas are presented in Tables 1 and 2. Daily water quality data collected during the assay are summarized in Table 3. Table 4 contains a summary of the data from the 120 hour *L. sativa* assay. Survival, growth and development data from the FETAX assay are summarized in Table 5. Data from the Microtox@ evaluation are provided in Table 6. A summary of reference toxicant data for the test species is provided in Table 7. Support data, including copies of laboratory bench sheets, are provided in Appendix A.

3.1 Chronic Toxicity Test - *Ceriodaphnia dubia*

Control survival on Day 7 was 90% with an average of 28.4 juveniles/female produced in the laboratory diluent control. At least 3 broods were produced by 60% of the laboratory diluent control females by the end of the assay. Minimum test acceptability criteria require 80% survival, mean production of 15 juveniles/female, and production of 3 broods by at least 60% of control females (EPA 1994). In addition, these values were within one standard deviation of the historic mean values for the laboratory control treatments. The results are an indication of healthy test organisms and document that the dilution water had no significant, adverse impact on the outcome of the assay.

After 48 hours exposure, survival was 100% in all tested concentrations. The 48 hour LC-50 was determined to be >10.0 mg/L sample with an associated A-NOEC value of 10.0 mg/L sample.

After 7 days exposure survival was >_70% in all test concentrations. Analysis of the data showed no statistically significant reduction in survival as compared to the control treatment. The C-NOEL for survival was determined to be 10.0 mg/L sample. The LOEC and chronic values were >10.0 mg/L sample.

Mean juvenile production in 10.0 mg/L sample was 23.8 juvenile/female. Mean juvenile production in the remaining sample treatments ranged from 18.73 juveniles/female in 0.3125 mg/L sample to 24.4 juveniles/female in 2.5 mg/L sample. Statistical comparisons made against the laboratory dilution water control showed that juvenile production in all sample treatments was not significantly lower than that observed in the laboratory diluent control. Based on these data, the C-NOEL for reproduction was determined to be 10.0 mg/L sample. The LOEC and chronic values were >10.0 mg/L sample.

3.2 Chronic Toxicity Test - *Pimephales promelas*

Control survival on Day 7 was 98% and the minnows had a mean dry weight, based on Day 7 survival, of 0.423 mg/fish in the laboratory diluent control. Minimum test acceptability criteria require 80% survival with a mean dry weight, based on Day 7 survival, of 0.250 mg/fish (EPA 1994). In addition, these values were within one standard deviation of the historic mean values for the laboratory control treatments. Mean dry weight/fish for the laboratory diluent control, based on Day 0 survival, was 0.414 mg/fish. This value was used for statistical comparisons. These data are an indication of healthy test organisms and that the dilution water had no adverse impacts on the outcome of the assay.

Survival after 48 hours exposure was 100% in all sample treatments. The 48 hour LC-50 was >10.0 mg/L sample with an A-NOEC of 10.0 mg/L sample. After 7 days exposure survival was >_90% in all sample treatments. Analysis of the data showed that survival in all sample concentrations was not significantly less than the laboratory diluent control. The C-NOEL for survival was 10.0 mg/L sample. The LOEC and chronic values were >10.0 mg/L sample.

The mean dry weight of surviving fish in 10.0 mg/L sample was 0.272 mg/fish. Mean dry weight of surviving fish in the diluted sample treatments ranged from 0.218 mg/fish in 5.0 mg/L sample to 0.359 mg/fish in 1.25 mg/L sample. Mean dry weight in 0.3125 mg/L, 2.5 mg/L, 5.0 mg/L and 10.0 mg/L sample treatments was significantly less than in the laboratory diluent control. The C-NOEL for growth was 1.25 mg/L sample. The LOEC value was 2.5 mg/L and the chronic value was 1.77 mg/L sample.

3.3 Seed Germination and Root Tip Elongation Assay

After 120 hours of exposure, 100% of the laboratory control seeds had germinated. Germination in all the test treatments was >_90%. The EC-50 value for germination was observed to be >10.0 mg/L sample with an associated NOEC of 10.0 mg/L sample.

The average root tip length in the control replicates was 36.1 mm. Root tip lengths in the tested concentrations ranged from 23.5 mm in 0.625 mg/L sample to 38.1 mm in 0.3125 mg/L sample. The Bonferroni t-Test indicated that the root tip length in 0.625 mg/L sample was significantly less than in the control replicates. The EC-50 value for root tip elongation was observed to be >10.0 mg/L sample with an associated NOEC of 10.0 mg/L sample.

3.4 FETAX Assay

At the end of the 96 hour exposure period, survival in the FETAX control (diluent) was 95% and 97% of the surviving tadpoles exhibited normal development. Tadpoles in the control replicates were an average of 9.90 mm in length. These values exceed the minimum acceptable criteria of 90% survival and 80% normal development specified by the protocol. Survival and normal development in the positive control, 6-aminonicotinamide, was within acceptable limits as specified by the protocol. These data indicate that the embryos used in the assay exhibited normal development and that the FETAX solution used in the preparation of the diluted sample concentrations had no impact on the outcome of the assay.

At the end of the 96-hour exposure period, survival ranged from 84% in 0.3125 mg/L sample to 96% 0.625 mg/L. Analysis of the data set showed that the survival data was normally distributed and the variances were not homogeneous. Based on these criteria, the data were evaluated using the Bonferroni t-Test. Results of the statistical evaluations showed that the survival of embryos exposed to all sample concentrations was not significantly different from the laboratory control. The LC-50 for survival was >10.0 mg/L sample with an associated NOEC of 10.0 mg/L sample.

Normal embryo development in the tested concentrations ranged from 90% in 5.0 mg/L sample to 100% in 0.625 mg/L sample. According to Dunn's Multiple Comparison Analysis, development in the tested concentrations was not significantly less than that in the control replicates. The EC-50 for normal development was >10.0 mg/L sample with an associated NOEC of 10.0 mg/L sample.

Tadpole lengths in the tested concentrations ranged from 9.49 mm in 10.0 mg/L sample to 10.0 mm in 2.5 mg/L sample. This data was normal and homogenous according to the TOXSTATO software. The Bonferroni t-Test indicated that tadpole length in 10.0 mg/L sample was less than that observed in the control replicates. The EC-50 for growth was >10.0 mg/L sample with an associated NOEC of 5.0 mg/L sample.

3.5 MicroToxO Evaluation

The 20 mg/L stock solution had an initial salinity of <1 ppt. The salinity adjusted stock solution had a pH of 6.03 SU and no visible turbidity. Results of the analysis show 5 and 15 minute EC-50 values of >10.0 mg/L sample.

3.6 Summary

The sample identified as S1-56 and provided by Aspen Systems Incorporated did not exhibit any signs of acute toxicity to the daphnid, *Ceriodaphnia dubia,* or the minnow, *Pimephales promelas,* during the initial 48 hour exposure period. The sample had an observed sublethal effect on minnow growth, but not on daphnid reproduction, during the 7 day exposure period. The sample had an effect on tadpole growth but not on tadpole survival or development. There was no observed effect on the survival or growth of the lettuce, L. *sativa,* or the performance of the
marine bacteria, V. *fischeri,* in the Microtox@ analysis.

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TABLE 1. Summary of Chronic Results: C. *dubia*. Aspen Product S1-56 Evaluation.

CHRONIC EXPOSURE EVALUATION, C. *dubia*

	SURVIVAL								JUVENILE PRODUCTION				
Sample	Exposure Period (Days)								Exposure Period (Days)				
Conc.0	0	1	2	3	4	5	6	7	3	4	5	6	7Total
Lab	10	10	10	10	10	9	9	9	0	32	24	51	177284
0.3125	10	10	10	10	8	8	8	8	0	8	20	21	138187
0.625	10	10	10	10	8	8	8	8	9	24	18	56	133240
1.25	10	10	10	10	7	7	7	7	0	30	8	28	130196
2.5	10	10	10	10	9	9	9	9	0	20	62	8	154244
5.0	10	10	10		10	10	9	9	0	31	40	10	123204
10.0	10	10	10	10	10	10	9	9	0	70	14	31	123238

	Lab	0.3125	0.625	1.25	2.5	5.0	10.0
Adult Survival, 7 days	90.0	80.0	80.0	70.0	90.0	90.0	90.0
Females Producing Young	90.0	80.0	80.0	70.0	90.0	100.0	100.0
Day of First Offspring	4	4	3	4	4	4	4
Mean No. of Offspring/Female	28.4	18.7	24.0	19.6	24.4	20.4	23.8

END POINT SUMMARY

		Chronic Exposure Evaluation		Acute Exposure Evaluation	
		Survival	Reproduction		
C-NOEL	=			10.0 mg/L	10.0 mg/L48 Hour
LC-50	=	>10.0 mg/L			
LOEC	=		>10.0 mg/L	>10.0 mg/L	48 Hour A-NOEC
	=	10.0 mg/L			
Chronic Value	=	>10.0 mg/L	>10.0 mg/L		

COMMENTS
0 - Concentrations expressed as mg/L product.

TABLE 2. Summary of Chronic Results: P. promelas. Aspen Product S1-56 Evaluation.

	SURVIVAL								GROWTH (mg/fish)"			
Sample	Exposure Period (Days)								Replicate			
Conc.0	0	1	2	3	4	5	6	7	A	B	C	D	Mean
Lab	40		40	40	40	40	39	39	39	0.439	0.431	0.355	0.4290.414
0.3125	40	40	40	40	40	40	40	40	0.304	0.369	0.398	0.224	0.324
0.625	40	40	40	39	38	38	38	38	0.301	0.351	0.345	0.316	0.328
1.25	40		40	40	40	40	40	40	40	0.325	0.284	0.390	0.4360.359
2.5	40		40	40	38	38	38	38	37	0.228	0.261	0.287	0.1680.236
5.0	40		40	40	40	39	36	36	36	0.232	0.193	0.172	0.2740.218
10.0	40		40	40	40	40	40	40	40	0.281	0.223	0.290	0.2930.272

	Lab	0.3125	0.625	1.25	2.5	5.0	10.0
Survival (%)	97.5	100.0	95.0	100.0	92.5	90.0	100.0
Growth (mg/fish)"	0.414	0.3241	0.328	0.359	0.2361	0.2181	0.2721

END POINT SUMMARY

	Chronic Exposure Evaluation		Acute Exposure Evaluation	
	Survival	Growth		
C-NOEL	=10.0 mg/L	2.5 mg/L	48 Hour LC-50 =	>10.0 mg/L
LOEC	=>10.0 mg/L	1.25 mg/L	48 Hour A-NOEC =	10.0 mg/L
Chronic Value	=>10.0 mg/L		1.77 mg/L	

COMMENTS:

- Growth calculations and statistics based on number of organisms added on Day 0.
  Significantly less than control treatment.

0 - Concentrations expressed as mg/L product.

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TABLE 3. Summary of Daily Water Quality Data C. *dubia* & *P, promelas.* Aspen Product S1-56 Evaluation.

PARAMETER	UNIT	Sample	LAB DILUENT	
Specific Conductance	pmhos/cm	Day 0	241	220
		Day 1	218	212
		Day 2	173	168
		Day 3	172	197
		Day 4	187	186
		Day 5	171	175
		Day 6	178	191
pH	SU	Day 0	7.83	7.58
		Day 1	7.95	7.77
		Day 2	7.85	7.93
		Day 3	7.88	7.77
		Day 4	7.87	7.75
		Day 5		
		Day 6	7.80	7.66
Alkalinity	mg/L	Day 0	10.1	28.8
Hardness	mg/L	Day 0	59	63
Total Solids	mg/L	Day 0	140	114
Total Suspended Solids	mg/L	Day 0	<10	<10
Total Residual Chlorine	mg/L	Day 0	<0.05	<0.05
Ammonia	mg/L	Day 0	<0.10	<0.10
Total Organic Carbon	mg/L	Day 0	0	0.28

COMMENTS
* Technician error. No data recorded.

TABLE 4. Summary of L. sativa Bioassay - Germination & Root Tip Lengths-0 Aspen Product S1-56 Evaluation.

Percent Germination

Species	Exposure	Lab	0.3125	0.625	1.25	2.5	5.0	10.0
L. sativa	120 Hours	100	100	90	100	100	94	95

Average Lengths (mm)

Species	Exposure	Lab	0.3125	0.625	1.25	2.5	5.0	10.0
L. sativa	120 hours	36.1	38.1	23.5	29.7	32.7	32.1	29.7

Bioassay Results

	Exposure	EC-50	NOEC
Germination	120 Hours	>10.0 mg/L	10.0
Growth	120 Hours	>10.0 mg/L	10.0

TABLE 5. Summary of FETAX Bioassay - Survival & Lengths.0 Aspen Product S1-56 Evaluation.

Percent Survival

Species	Exposure	Lab	0.3125	0.625	1.25	2.5	5.010.0
X. laevis	96 hours	95	84	96	94	90	9490

Percent Normal Development

Species	Exposure	Lab	0.3125	0.625	1.25	2.5	5.0	10.0
X. laevis	96 hours	97	96	100	96	95	90	91

Average Lengths (mm)

Species	Exposure	Lab	0.3125	0.625	1.25	2.5	5.0	10.0
X. laevis	96 hours	9.90	9.89	9.86	9.87	10.00	9.82	9.491

Bioassay Results

	Exposure	**LC-50 or EC-50**	**NOEC**
SURVIVAL	120 HOURS	>10.0 MG/L	10.0 MG/L
Development	120 Hours	>10.0 mg/L	10.0 mg/L
Growth	120 Hours	>10.0 mg/L	5.0 mg/L

COMMENTS

0 - Concentrations expressed as mg/L product.

TABLE 6. Summary of Microtox Analysis.
Aspen Product S7-56 Evaluation.

EC-50	Time (min)	TRC (mg/L)	As rec'd Salinity (ppt)	Adjusted Salinity (ppt)	As rec'd pH (SU)
>10.0 mg/L	5	<0.05	<1	18	6.03
>10.0 mg/L	15	<0.05	<1	18	6.03

TABLE 7. Summary of Reference Toxicant Data.
Aspen Product S1-56 Evaluation.

Species	Endpoint	Result	Units	Historic Mean	No. of Tests	STD Deviations	
C. dubia	48 Hr LC-50	26.4	mg/L SDS	16.7	18	9.21	18.4
P. promelas	48 Hr LC-50	27.0	mg/L SIDS	23.5	17	6.70	13.4
L. sativa	120 Hr NOEC	0.10	mg/L Cd	0.373		0.55	1.09
L. sativa	120 Hr IC-25	0.77	mg/L Cd	1.11	3	1.12	2.24
X. laevis	96 Hr EC-50	7.97	mg/LO	7.97	1	0	0

Species -	Endpoint	Result	Units	Acceptable
V. fisheri	5 min LC-50	16.2	mg/L Phenol	13 - 26
V. fisheri	15 min LC-50	3.8	mg/L Zn	3 - 10

o - mg/L 6-aminonicotinamide.

Task 9. Scale-up Expression and Purification of the Mutated *D. can.* Antifreeze Protein.

Collaborative BioAlliance Inc. has been contracted and is currently developing the scale-up production and purification systems of the mutant *D. can.* antifreeze proteins. On going analysis of the purified protein is determining the direction of the fermentation parameters.

To date there have not been any technical or peer-reviewed papers published on this project todate. Preparation on patent applications for the mutated *Dendroides canadenesis* AFPs is underway.